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(54) Title: **NOVEL TOXINS**

(57) Abstract: Novel bacterial insecticidal proteins and equivalents thereof were isolated. These proteins and the DNA sequences encoding them are useful to make insecticidal compositions or transgenic plants to protect plants from damage by insects, particularly coleopteran insects.

WO 01/87931 A2

NOVEL TOXINS

Background of the Invention5 1. Field of the Invention

The present invention relates to new insecticidal secreted proteins ("ISPs") isolated from a bacterial strain, preferably a Brevibacillus species strain, most preferably a Brevibacillus laterosporus species strain which are insecticidal when ingested in combination with an ISP-complimentary protein such as another ISP protein of this invention, and to DNA sequences
10 encoding such proteins. These proteins are useful to prevent or minimize insect damage, particularly of corn rootworms, to plants in a field.

The present invention also relates to plants, particularly corn plants, that are rendered
15 insecticidal, preferably to coleopteran insects, particularly to Diabrotica spp., Leptinotarsa spp. and Anthonomus species insects, by the expression of the ISP proteins of this invention in cells of said plants.

The present invention also relates to a method for controlling damage by Diabrotica spp.,
20 Leptinotarsa spp. or Anthonomus species insects, preferably Diabrotica spp. insect pests, particularly corn rootworms, by having the ISP proteins of the invention, particularly the proteins with the amino acid sequence of any one of SEQ ID No. 2, 4, 8 or 10, or insecticidally-effective fragments thereof, ingested by said insects.

2. Description of the Prior Art

Some of the most destructive pests are found among the Diabroticine beetles. In North America, the three important species of corn rootworms, Diabrotica virgifera (the Western corn rootworm), Diabrotica barberi (the Northern corn rootworm) and Diabrotica undecimpunctata howardi (the Southern corn rootworm) are considered to be the most expensive insect pests to control (Metcalf, 1986, Foreword in "Methods for the Study of Pest Diabrotica", pp. vii-xv, eds. Krysan, J.L. and Miller, T.A., Springer-Verlag, New York). Diabrotica virgifera and Diabrotica barberi are considered the most serious insect pests of corn in the major corn-producing states of the United States and Canada (Levine and Oloumi-Sadeghi, 1991, Annu. Rev. Entomol. 36, 229-55). The larvae feed on the roots and thus cause direct damage to corn growth and corn yields. Costs for soil insecticides to control larval damage to the root systems of corn and aerial sprays to reduce beetle damage to corn silks, when combined with crop losses, can approach one billion dollars annually (Metcalf, 1986, supra). Recently, in some US states it was discovered that the crop rotation program of planting soybeans after corn lost its effect as corn rootworms have adapted to this situation.

Bacterial strains and/or genes with toxicity to corn rootworm have been described in US patents 6,023,013; 6,015,553; 6,001,637; 5,906,818; and 5,645,831. Also, PCT publications WO 00/09697, WO 99/57282, WO 98/18932, WO 97/40162, and WO 00/26378 relate to toxins and genes obtainable from Bacillus or other bacterial spp., some of which are described to have toxicity to corn rootworm. WO 98/44137, WO 94/21795 and WO 96/10083 relate to pesticidal Bacillus strains, characterized by pesticidal proteins and auxiliary proteins produced during vegetative growth, some of which are described to have toxicity to corn rootworm. US patent 5,055,293 describes a method to control corn rootworms by inoculating soil with parasporal-inclusion forming species of Bacillus laterosporus.

Orlova et al. (1998, Applied Environmental Microbiol. 64, 2723) showed insecticidal activity to mosquitoes associated with protein crystals in crystal-forming strains of Bacillus laterosporus.

Objects and Summary of the Invention

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An object of the present invention is to provide novel proteins and DNA sequences encoding such proteins with significant toxicity to insects, preferably Diabrotica spp. insects, particularly corn rootworm.

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In one embodiment of the invention, a protein is provided comprising the amino acid sequence of the smallest active toxin of the protein of SEQ ID No. 2, wherein said smallest active toxin is:

a) a fragment of the protein of SEQ ID No. 2, and

b) insecticidal to Diabrotica virgifera larvae when ingested by said larvae in combination with the protein of SEQ ID No. 4 from amino acid position 51 to 457. Also provided is a protein

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comprising the amino acid sequence of the smallest active toxin of the protein of SEQ ID No. 4, wherein said smallest active toxin is:

a) a fragment of the protein of SEQ ID No. 4, and

b) insecticidal to Diabrotica virgifera larvae when ingested by said insect in combination with the protein of SEQ ID No. 2 from amino acid position 38 to 871.

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Particularly preferred is a protein characterized by an amino acid sequence comprising the sequence of SEQ ID No. 2 from amino acid position 38 to amino acid position 768 or 781, preferably the protein characterized by the amino acid sequence of SEQ ID No. 2 or SEQ ID No. 10; and a protein characterized by an amino acid sequence comprising the sequence of

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SEQ ID No. 4 from amino acid position 51 to amino acid position 449 or 457, preferably a protein characterized by the amino acid sequence of SEQ ID No. 4 or SEQ ID No. 8.

A further object of the invention is a protein comprising the amino acid sequence of the protease-digestion fragment of the protein encoded by the isp1A DNA deposited at the BCCM-LMBP under accession number LMBP 4009, which protease-digestion fragment is insecticidal to Diabrotica virgifera upon combined application with the protein of SEQ ID. No. 4 from amino acid position 51 to amino acid position 457; and a protein comprising the amino acid sequence of the protease-digestion fragment of the protein encoded by the isp2A DNA deposited at the BCCM-LMBP under accession number LMBP 4009, which protease-digestion fragment is insecticidal to Diabrotica virgifera upon combined application with the protein of SEQ ID No. 2 from amino acid position 38 to amino acid position 871; particularly wherein said protease-digestion fragment is obtainable by treatment with coleopteran gut juice.

Also provided in accordance with this invention is a DNA sequence encoding the above proteins, particularly a DNA comprising an artificial DNA sequence having a different codon usage compared to the naturally occurring DNA sequence but encoding the same protein sequence, preferably contained in a chimeric gene operably linked to a plant-expressible promoter region (i.e., a promoter region which is suitable for expression in plant cells, this can be from bacterial, viral or plant origin or can be artificially made); particularly a promoter region which is preferentially active in root tissue.

In one embodiment of the invention, the promoter in said chimeric gene comprises the DNA sequence of SEQ ID No. 5 or 6 or a DNA hybridizing thereto under stringent hybridization conditions.

In a further embodiment of this invention, the chimeric gene further comprises a signal peptide for secretion from the cell or for targeting to a cellular organelle, particularly a chloroplast transit peptide.

Also provided is a plant cell, a plant or a seed, comprising any of these chimeric genes integrated in their cells, particularly a combination of the chimeric gene encoding the ISP1A, or an insecticidally effective fragment thereof, and the chimeric gene encoding the ISP2A protein, or an insecticidally effective fragment thereof; particularly a corn cell, plant or seed.

In another embodiment of this invention, a micro-organism transformed to contain any of the above DNA sequences is provided.

- Also provided is a process for controlling insects, particularly a process for rendering a plant resistant to coleopteran insects, comprising expressing any of the ISP proteins of this invention in cells of a plant, and regenerating transformed plants from said cells which are resistant to insects. In such process, the insect is preferably selected from the group consisting of: rootworms, weevils, potato beetles, Diabrotica species, Anthonomus spp., Leptinotarsa spp., Agelastica alni, Hypera postica, Hypera brunneipennis, Haltica tombacina, Anthonomus grandis, Tenebrio molitor, Triboleum castaneum, Dicladispa armigera, Trichispa serica, Oulema oryzae, Colaspis brunnea, Lissorhoptrus oryzophilus, Phyllotreta cruciferae, Phyllotreta striolata, Psylliodes punctulata, Entomoscelis americana, Meligethes aeneus, Ceutorynchus sp., Psylliodes chrysocephala, Phyllotreta undulata, Leptinotarsa decemlineata, Diabrotica undecimpunctata undecimpunctata, Diabrotica undecimpunctata howardi, Diabrotica barberi, and Diabrotica virgifera.

Yet another object of the present invention is to provide a method for rendering plants insecticidal against Coleoptera and a method for controlling Coleoptera, comprising planting, sowing or growing in a field plants transformed with DNA sequences encoding the ISP proteins of the invention, particularly corn plants. In an embodiment of this invention, the ISP proteins

are combined with other insecticidal proteins or protein combinations, preferably corn rootworm-toxic proteins.

Also provided in accordance with this invention are ISP1A or ISP2A equivalents, preferably from a Brevibacillus laterosporus strain, particularly from a Brevibacillus laterosporus strain not forming crystalline inclusions. Such equivalents preferably have molecular weights of about 95 to about 100 kD for ISP1A equivalents, and about 45 to about 50 kD for ISP2A equivalents, as determined in standard 8-10 % SDS-PAGE gel electrophoresis using appropriate molecular weight markers.

Detailed Description of the Preferred Embodiments of the Invention

In accordance with this invention, new bacterial toxins and DNA sequences encoding them have been isolated and characterized. The new proteins were designated ISP1A and ISP2A, and the DNA sequences encoding them isp1A and isp2A.

In accordance with this invention "ISP1A protein" refers to any protein comprising the smallest protein fragment of the amino acid sequence of SEQ ID No. 2 which retains insecticidal activity, particularly to coleopteran insects, more particularly to corn rootworm, cotton boll weevil and Colorado potato beetle, preferably to Diabrotica spp., especially to Southern, Western and Northern corn rootworm, particularly to Diabrotica virgifera, upon combined ingestion by an insect with a suitable ISP-complimentary protein, particularly the mature ISP2A protein. This includes any protein with an amino acid sequence comprising the amino acid sequence from the amino acid at position 32 or 38, preferably 38, to an amino acid at a position from amino acid position 768 to amino acid position 871 in SEQ ID No. 2, particularly any protein with an amino acid sequence comprising at least the amino

acid sequence of SEQ ID No. 2 from amino acid position 38 to amino acid position 768. This also includes the protein obtained from the amino acid sequence of SEQ ID No. 2 by cleaving off part or all of the N-terminal signal peptide sequence or the protein wherein the signal peptide sequence has been replaced by another, e.g. a plant, targeting peptide, a methionine amino acid or a methionine-alanine dipeptide. Specifically, this includes the protein comprising an N-terminal prokaryotic or eucaryotic, e.g. bacterial or plant, signal peptide for secretion or targeting. This also includes hybrids or chimeric proteins comprising the above described smallest toxic protein fragment, e.g., a hybrid between an ISP1A and ISP2A protein of this invention. Further, included in the designation "ISP1A", as used herein, are also protease-resistant fragments of the ISP1A protein retaining insecticidal activity obtainable by treatment with insect gut juice, preferably coleopteran gut juice, particularly coleopteran gut proteases, preferably corn rootworm proteases, e.g., cysteine proteinases, serine proteinases, trypsin, chymotrypsin or trypsin-like proteases. Particularly, the coleopteran is selected from the group consisting of: corn rootworm, cotton boll weevil and Colorado potato beetle, Diabrotica spp., Diabrotica virgifera, Diabrotica barberi, Diabrotica undecimpunctata, Leptinotarsa decemlineata, and Anthonomus grandis. In a preferred embodiment of this invention, an ISP1A protein according to this invention is not insecticidal when ingested in isolation without providing simultaneously or sequentially, an ISP complimentary protein such as an ISP2A protein.

In accordance with this invention "ISP2A protein" refers to any protein comprising the smallest protein fragment of the amino acid sequence of SEQ ID No. 4 which retains insecticidal activity, particularly to coleopteran insects, more particularly to corn rootworm, cotton boll weevil and Colorado potato beetle, preferably to Diabrotica spp., especially to Diabrotica virgifera, Diabrotica barberi, Diabrotica undecimpunctata, upon combined ingestion by an insect with a suitable ISP-complimentary protein, particularly the mature

ISP1A protein. This includes any protein with an amino acid sequence comprising the amino acid sequence from the amino acid at position 43 or 51, preferably 51, to the amino acid at a position from amino acid position 449 to position 457 in SEQ ID No. 4, particularly any protein with an amino acid sequence comprising at least the amino acid sequence of SEQ ID No. 4 from amino acid position 51 to position 449. This also includes the protein obtained from the amino acid sequence of SEQ ID No. 4 by cleaving off part or all of the N-terminal signal peptide sequence of the protein, and the protein wherein the signal peptide has been replaced by another, e.g., a plant, targeting peptide, a methionine amino acid or a methionine-alanine dipeptide. Specifically, this includes the protein comprising an N-terminal prokaryotic or eucaryotic, e.g. bacterial or plant, signal peptide for secretion or targeting. This also includes hybrids or chimeric proteins comprising the above described smallest toxic protein fragment, e.g., a hybrid between an ISP1A and ISP2A protein of this invention. Further, included in the designation "ISP2A", as used herein, are also protease-resistant fragments of the ISP2A protein retaining insecticidal activity obtainable by treatment with insect gut juice, preferably coleopteran gut juice, particularly coleopteran gut proteases, e.g., cysteine proteinases, serine proteinases, trypsin, chymotrypsin or trypsin-like proteases. In a preferred embodiment of this invention, an ISP2A protein according to this invention is not insecticidal when ingested in isolation without providing simultaneously or sequentially, an ISP complimentary protein such as an ISP1A protein.

An "ISP-complimentary protein", as used herein, refers to a protein, including but not limited to the mature ISP1A or ISP2A protein, which in combination with one of the ISP proteins of this invention, is insecticidal upon ingestion by an insect, particularly a coleopteran insect, preferably a corn rootworm, a cotton boll weevil or Colorado potato beetle, more particularly Diabrotica virgifera, Diabrotica barberi, Diabrotica undecimpunctata or Anthonomus grandis. Particularly, also VIP proteins and active fragments thereof, particularly the mature VIP

proteins with their signal sequences cleaved off, as described in WO 98/44137, WO 94/21795 and WO 96/10083 are ISP-complimentary proteins in accordance with this invention. An ISP-complimentary protein to the ISP1A protein is ideally the ISP2A protein or the VIP2Aa or VIP2Ab protein or active fragments thereof (such as the mature proteins with the signal sequences removed) as described in US patent 5,990,383, or any bacterial
5 secreted protein which has a sequence identity of at least 50 %, preferably at least 75 %, particularly at least 85 %, to any one of the ISP2 or VIP2 proteins, and which is insecticidal when ingested by an insect, preferably a coleopteran insect, particularly a corn rootworm, in combination with the mature ISP1A protein. An ISP-complimentary protein to the ISP2A
10 protein is ideally the mature ISP1A protein, the VIP1Aa or VIP1Ab protein or active fragments thereof (such as the mature protein with the signal peptide removed) as described in US patent 5,990,383, or any bacterial secreted protein which has a sequence identity of at least 50 %, preferably at least 75 %, particularly at least 85 %, to any one of the ISP1 or
VIP1 proteins, and which is insecticidal when ingested by an insect, preferably a coleopteran
15 insect, particularly a corn rootworm, in combination with the mature ISP2A protein. For the avoidance of doubt, an ISP-complimentary protein and an ISP protein are always different proteins.

In a preferred embodiment of this invention, the ISP proteins of this invention, or their
20 equivalents, when used in isolation, i.e., without any of the complementary ISP proteins present, do not result in any significant insecticidal activity, preferably to corn rootworm larvae, particularly to Diabrotica virgifera, when tested in a surface contamination assay on standard insect diet, and this at a concentration wherein the proteins (each applied in that concentration) in combination result in 100% mortality, preferably to corn rootworm larvae,
25 particularly to Diabrotica virgifera. Particularly, the ISP1 proteins of this invention give no significant mortality (i.e., no difference with the controls using a buffer solution alone) to

Diabrotica virgifera larvae when only an ISP1 protein is applied at a concentration of 70ng/cm² in a surface contamination assay using standard corn rootworm diet, and the ISP2 proteins of this invention give no significant mortality (i.e., no difference with the controls using a buffer solution alone) to Diabrotica virgifera larvae when only an ISP2 protein is applied at a concentration of 36 ng/cm² in a surface contamination assay using standard corn rootworm diet, while ISP1 and ISP2 proteins applied together in these concentrations in the same type of assay give 100 % mortality to these larvae.

As used herein, "ISP1A equivalent" or "ISP2A equivalent" refers to a protein with the same or substantially the same toxicity to a target insect as the ISP1A or ISP2A protein, respectively, when applied to such target insect, preferably when ingested by such insect, in a binary combination with an ISP-complimentary protein, and with substantially the same amino acid sequence as the ISP1A or ISP2A protein, respectively. Also included in the definition of ISP1A or ISP2A equivalents are bacterial proteins of respectively about 45 to about 50 kD and about 95 to about 100 kD molecular weight as determined by standard 8-10 % SDS-PAGE gel electrophoresis, preferably from a Brevibacillus laterosporus strain, particularly from a Brevibacillus laterosporus strain not forming crystalline inclusions, which proteins in combination but not in isolation, have significant insecticidal activity to corn rootworm larvae.

The use of the terms "in combination", when referring to the application of an ISP protein (or its equivalent) and an ISP-complimentary protein to a target insect to get an insecticidal effect, includes the simultaneous application (i.e., in the same feed, cells or tissue and applied or ingested by an insect at the same moment) and the separate, sequential application (i.e. one is provided after the other but not applied or ingested at the same time) of an ISP protein (or its equivalent) and an ISP-complimentary protein of this invention, as long as these proteins are found together in the insect gut at one moment in time. The

ISP1A protein of this invention could thus be expressed in a certain type of cells or a certain zone in the roots of a plant, while the ISP2A protein of this invention could be expressed, in another kind of cells or another zone in the roots of the same plant, so that the proteins will only interact once root material is ingested. Also included herein is the expression of an ISP protein or its equivalent in roots of a plant, particularly a corn plant, and the expression of an ISP-complimentary protein in root-associated bacteria such as rhizobacteria strains (or vice versa).

"The same toxicity to a target insect", with respect to an ISP protein and an ISP equivalent protein as used herein, means that the mean mortality of the ISP protein, in the presence of a suitable ISP-complimentary protein, is not significantly different from the mean mortality of the ISP equivalent, also in the presence of the same suitable ISP-complimentary protein. Particularly, this refers to the situation wherein the 95% fiducial limits of the LC50 of the ISP protein (when tested in the presence of a suitable ISP-complimentary protein) overlap with the 95% fiducial limits of the LC50 of the ISP equivalent (when tested in the presence of a suitable ISP-complimentary protein).

"Substantially the same toxicity to a target insect", as used herein with respect to an ISP protein and an ISP equivalent protein, refers to levels of mean mortality of such proteins to a target insect which are significantly different between the ISP and the ISP equivalent protein, but which are still within a range of insecticidal activity which is useful to control or kill the relevant target insect, preferably when such protein is expressed in a plant. In a preferred embodiment of this invention, proteins have substantially the same toxicity to a target insect when their LC50 values for such a target insect in the same (replicated) in vitro assay conducted under the same assay conditions differ from each other by a factor 2 to 100, preferably 2 to 50, particularly 2 to 20, most preferably 2 to 10.

Also, functionally analogous amino acids can be used to replace certain amino acids in an ISP1A or ISP2A protein to obtain ISP1A or ISP2A equivalents. For example, one or more amino acids within the sequence can be substituted by other amino acids of a similar polarity which act as a functional equivalent, resulting in a silent alteration with respect to functionality of the protein. Substitutes for an amino acid within the sequence can be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Proteins wherein conservative amino acids replacements are made using amino acids of the above indicated same class which retain substantially the same insecticidal activity to a target insect compared to the original protein, are included herein as equivalents of the ISP proteins of this invention.

A protein with "substantially the same amino acid sequence" to an ISP1A protein, as used herein, refers to a protein with at least 90 %, particularly at least 95 %, preferably at least 97 % sequence identity with the ISP1A protein, wherein the percentage sequence identity is determined by using the blosum62 scoring matrix in the GAP program of the Wisconsin package of GCG (Madison, Wisconsin, USA) version 10.0 (GCG defaults used). "Sequence identity", as used throughout this application, when related to proteins, refers to the percentage of identical amino acids using this specified analysis. The "sequence identity", as used herein, when related to DNA sequences, is determined by using the nwsgapdna scoring matrix in the GAP program of the Wisconsin package of GCG (Madison, Wisconsin, USA) version 10.0 (GCG defaults used).

"ISP protein" or "ISP protein of this invention", as used herein, refers to any one of the new proteins of this invention and identified herein as ISP1A or ISP2A protein or their equivalents. An "ISP protoxin" refers to the full length ISP1A or ISP2A protein as it is encoded by the naturally-occurring bacterial DNA sequence, including the signal peptide.
5 An "ISP toxin" refers to an insecticidal fragment thereof, particularly the smallest toxic fragment thereof or the mature protein with the signal peptide removed. A "mature" ISP, as used herein, refers to the ISP protein of this invention as secreted by its native bacterial host cell which is insecticidally active in combination with an ISP-complimentary protein (without the N-terminal bacterial signal peptide sequence). A mature ISP protein can have a
10 complete native C-terminal or can have a C-terminal truncation.

Also included in this invention as an ISP protein is a first protein with an apparent molecular weight of its mature form of about 95 to about 100 kD, particularly about 100 kD, which is
15 secreted by a bacterium, preferably a bacterium which is not B. thuringiensis or B. cereus, particularly a bacterium which is Brevibacillus laterosporus, or insecticidally effective fragments of said first protein, characterized by a significant insecticidal activity when combined with a second protein with an apparent molecular weight of its mature form of about 45 to about 50 kD, preferably about 45 kD, which is secreted by a bacterium, preferably a bacterium which is
20 not B. thuringiensis or B. cereus, particularly a bacterium which is Brevibacillus laterosporus, or insecticidally effective fragments of said second protein, wherein the combination of said first and said second protein, or their insecticidal fragments, is significantly insecticidal to larvae of the Colorado potato beetle, the Western corn rootworm, the Southern corn rootworm, and the Northern corn rootworm, when ingested by said insects, and wherein said protein combination
25 is not significantly insecticidal to Ostrinia nubilalis, Spodoptera frugiperda, Heliiothis virescens, Helicoverpa zea and Sesamia nonagroides, when ingested by said insects, and wherein said

first protein alone has no significant insecticidal activity when ingested by an insect. Also included herein is the second protein as described above which is insecticidal when ingested by an insect in combination with the above described first protein.

5 "Apparent molecular weight", as used herein, is the molecular weight as evidenced by SDS-PAGE analysis upon comparison with molecular weight standards. As is appreciated by the skilled person, this molecular weight is an approximate determination and can have a range of variation of about 10-15 % with respect to the actual molecular weight as determined based on the amino acid sequence.

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As used herein, the terms "isp1A DNA" or "isp2A DNA" refer to any DNA sequence encoding the ISP1A or ISP2A protein, respectively, as defined above. This includes naturally occurring, artificial or synthetic DNA sequences encoding the newly isolated proteins or their fragments as defined above, particularly DNA sequences with a modified codon usage adapted to more closely match the codon usage of a plant. Examples of artificial DNA sequences encoding an ISP1A and ISP2A protein are shown in SEQ ID Nos. 9 and 7, respectively (the ISP proteins encoded by these DNA sequences are defined herein as ISP1A-1 and ISP2A-1). Also included herein are DNA sequences encoding insecticidal proteins which are similar enough to the DNA sequence of SEQ ID No. 1, 3, 7 or 9 so that they can (i.e., have the ability to) hybridize to these DNA sequences under stringent hybridization conditions. "Stringent hybridization conditions", as used herein, refers particularly to the following conditions wherein hybridization is still obtained: immobilizing a first DNA sequence on filters, and prehybridizing the filters for either 1 to 2 hours in 50 % formamide, 5 % SSPE, 2x Denhardt's reagent and 0.1 % SDS at 42 ° C or 1 to 2 hours in 6x SSC, 2xDenhardt's reagent and 0.1 % SDS at 68 °C. The denatured radiolabeled second DNA is then added directly to the prehybridization fluid and incubation is carried out for 16 to

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24 hours at one of the above selected temperatures. After incubation, the filters are then washed for 20 minutes at room temperature in 1x SSC, 0.1 % SDS, followed by three washes of 20 minutes each at 68 °C in 0.2x SSC and 0.1 % SDS. An autoradiograph is established by exposing the filters for 24 to 48 hours to X-ray film (Kodak XAR-2 or equivalent) at -70 °C with an intensifying screen. Of course, equivalent conditions and parameters can be used in this process while still retaining the desired stringent hybridization conditions. As used herein, stringent hybridization preferably occurs between DNA sequences with at least 90 to 95 %, preferably at least 97 %, particularly 99%, sequence identity.

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Also included in the definition of "isp1 DNA" are all DNA sequences encoding a protein with a sequence identity of at least 90 %, preferably at least 95 %, particularly at least 97 %, most preferably at least 99 % with the protein of SEQ ID. No. 2 and which has substantially the same, preferably the same, insecticidal activity of the protein of SEQ ID No. 2, wherein said protein sequence identity is determined by using the blosum62 scoring matrix in the GAP program of the Wisconsin package of GCG (Madison, Wisconsin, USA) version 10.0 (GCG defaults used). Included in the definition of "isp2 DNA" are all DNA sequences encoding a protein with a sequence identity of at least 90 %, preferably at least 95 %, particularly at least 97 %, most preferably at least 99 %, with the protein of SEQ ID. No. 4 and which has substantially the same, preferably the same, insecticidal activity of the protein of SEQ ID No. 4, wherein said protein sequence identity is determined by using the blosum62 scoring matrix in the GAP program of the Wisconsin package of GCG (Madison, Wisconsin, USA) version 10.0 (GCG defaults used).

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An "isp gene" or "isp DNA", as used herein, is a DNA sequence encoding an ISP protein in accordance with this invention, referring to any one of the isp1A or isp2A DNA sequences defined above.

- 5 An "isp DNA equivalent" or an "isp gene equivalent", as used herein, is a DNA encoding an ISP equivalent protein as defined above.

The terms "DNA/protein comprising the sequence X" and "DNA/protein with the sequence comprising sequence X", as used herein, refer to a DNA or protein including or containing at least the sequence X in their nucleotide or amino acid sequence, so that other nucleotide or amino acid sequences can be included at the 5' (or N-terminal) and/or 3' (or C-terminal) end, e.g., an N-terminal transit or signal peptide. The term "comprising", as used herein, is open-ended language in the meaning of "including", meaning that other elements than those specifically recited can also be present. The term "consisting of", as used herein, is closed-ended language, i.e., only those elements specifically recited are present. The term "DNA encoding a protein comprising sequence X", as used herein, refers to a DNA comprising a coding sequence which after transcription and translation results in a protein containing at least amino acid sequence X. A DNA encoding a protein need not be a naturally-occurring DNA, and can be a semi-synthetic, fully synthetic or artificial DNA and can include introns and 5' and/or 3' flanking regions. The term "nucleotide sequence", as used herein, refers to the sequence of a DNA or RNA molecule, which can be in single- or double-stranded form.

The term "gene", as used herein refers to a DNA coding region flanked by 5' and/or 3' regulatory sequences allowing an RNA to be transcribed which can be translated to a protein, typically comprising at least a promoter region. A "chimeric gene", when referring to an isp DNA of this invention, refers to an isp DNA sequence having 5' and/or 3' regulatory

sequences different from the naturally-occurring bacterial 5' and/or 3' regulatory sequences which drive the expression of the ISP protein in its native host cell.

"Insecticidal activity" or "insecticidally effective", when referring to an ISP protein of the invention or its equivalents, as used herein, means the capacity of an ISP protein to kill insects above the levels found in control treatment under the same assay conditions, upon the ingestion of such protein by an insect, preferably a coleopteran insect, particularly a corn rootworm, especially Diabrotica virgifera, in combination with an ISP-complimentary protein, such as a second ISP protein. Preferably the second ISP protein is the other protein encoded by the same bacterial operon as the first ISP or an insecticidally-effective fragment or equivalent thereof, yielding optimal insect mortality upon ingestion of the combined proteins. "Insect-controlling amounts" of an ISP protein, as used herein, refers to an amount of ISP protein which is sufficient to limit damage on a plant by insects feeding on such plant to commercially acceptable levels, e.g. by killing the insects or by inhibiting the insect development or growth in such a manner that they provide less damage to a plant and plant yield is not significantly adversely affected, when such ISP protein is provided with an ISP-complimentary protein, such as another ISP protein, preferably the other protein encoded by the same operon as the first ISP, or an insecticidally effective fragment thereof. "Insecticidally-effective ISP fragment", as used herein, refers to a fragment of an ISP protein of this invention which retains insecticidal activity when provided in combination with an ISP-complimentary protein, such as another ISP protein, preferably the other ISP encoded by the same operon as the first ISP. In the above definitions related to insecticidal activity, the insect preferably is a larva in any of the larval stages. Throughout this application, reference can be made to an "isp DNA and insecticidally-effective fragments or equivalents thereof", and in that case the insecticidal activity obviously refers to the activity of the protein encoded by the DNA and not to the insecticidal activity of the DNA itself.

In accordance with this invention, the ISP proteins of this invention and their equivalents, particularly the mature ISP1A and ISP2A proteins, were found to have no significant insecticidal activity to lepidopteran insects selected from the group consisting of: Heliothis virescens, Helicoverpa zea, Manduca sexta, Helicoverpa armigera, Spodoptera littoralis,
5 Spodoptera frugiperda, Sesamia nonagroides, and Ostrinia nubilalis.

In accordance with this invention, target insects susceptible to the ISP proteins of the invention or their equivalents are contacted with these proteins in insect-controlling amounts,
10 preferably insecticidal amounts, by expression in a transgenic plant of DNA sequences encoding such ISP proteins. Said target insects will only be affected by the insecticidal proteins when they ingest plant tissue. Thus, in another object of the present invention a method is provided for rendering plants insecticidal against Coleoptera and a method for controlling Coleoptera, comprising planting, sowing or growing in a field plants transformed with
15 DNA sequences encoding the ISP proteins of the invention, particularly corn plants.

The signal peptide of the ISP proteins of the invention can be removed or modified according to procedures known in the art, see, e.g., published PCT patent application WO 96/10083, or they can be replaced by another peptide such as a chloroplast transit peptide (e.g., Van Den
20 Broeck et al., 1985, Nature 313, 358, or preferably the modified chloroplast transit peptide of US patent 5, 510,471) causing transport of the protein to the chloroplasts, by a secretory signal peptide or a peptide targeting the protein to other plastids, mitochondria, the ER, or another organelle, or it can be replaced by a methionine amino acid or by a methionine-alanine dipeptide. Signal sequences for targeting to intracellular organelles or for secretion
25 outside the plant cell or to the cell wall are found in naturally targeted or secreted proteins, preferably those described by Klös gen et al. (1989, Mol. Gen. Genet. 217, 155-161), Klös gen

and Weil (1991, Mol. Gen. Genet. 225, 297-304), Neuhaus & Rogers (1998, Plant Mol. Biol. 38, 127-144), Bih et al. (1999, J. Biol. Chem. 274, 22884-22894), Morris et al. (1999, Biochem. Biophys. Res. Commun. 255, 328-333), Hesse et al. (1989, EMBO J. 8 2453-2461), Tavladoraki et al. (1998, FEBS Lett. 426, 62-66), Terashima et al. (1999, Appl. Microbiol. Biotechnol. 52, 516-523), Park et al. (1997, J. Biol. Chem. 272, 6876-6881), Shcherban et al. (1995, Proc. Natl. Acad. Sci USA 92, 9245-9249), all of which are incorporated herein by reference, particularly the signal peptide sequences from targeted or secreted proteins of corn. Although a DNA sequence encoding such a plant signal peptide can be inserted in the chimeric gene encoding the ISP1A and in the chimeric gene encoding the ISP2A protein for expression in plants, in one embodiment of this invention, a DNA sequence encoding such a signal peptide is only inserted in the chimeric ISP2A gene and the ISP1A chimeric gene lacks a signal peptide, or has a methionine amino acid or a methionine-alanine dipeptide instead of the signal peptide. In a preferred embodiment of this invention, the proteins are secreted from the roots as described by Gleba et al. (1999, Proc. Natl. Acad. Sci. USA 25, 5973-5977) and Borisjuk et al., 1999 (Nat. Biotechn. 17, 466-469), using a strong root-preferred promoter, particularly a strong root-preferred promoter from corn, and an N-terminal signal peptide for secretion from the root cells, preferably from corn, particularly the signal peptide of a secreted protein, preferably a corn secreted protein, selected from the group of: expansin, alpha-amylase, polyamine oxidase, cytokinin oxidase, endoxylanase.

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Plants included in the scope of this invention are corn, cotton, soybean, peas, beans, lentils, potato, tomato, tobacco, lettuce, Brassica species plants, sugarcane, rice, oilseed rape, mustard, asparagus, wheat, barley, coffee, tea, vines, rubber plant, beet, turf grasses, sorghum, oats, rye, onions, carrots, leek, cucumber, squash, melon, sunflower, particularly any plant which is susceptible to damage by coleopteran insects, preferably corn rootworm, potato beetles or weevils, particularly insects of the Diabrotica or Leptinotarsa species, most

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preferably any insect selected from the group consisting of: Diabrotica virgifera, Diabrotica barberi, Diabrotica undecimpunctata, Leptinotarsa decemlineata and Anthonomus grandis, most preferably corn plants.

5 "Corn", as used herein, refers to all plants of the species Zea mays, and any seeds, roots or grain, or other materials containing or directly produced from corn cells, of any variety of Zea mays, including but not limited to field corn, sweet corn, hybrid corn, white corn and dent corn, whether hybrid or inbred lines. Preferably, the corn plants used in this invention are suitable parent lines for producing hybrid corn, and most preferably they already carry an
10 endogenous or transgenic insect-resistance giving them protection from the major corn lepidopteran insect pests including but not limited to Ostrinia nubilalis.

The ISP1A and ISP2A proteins of this invention can be isolated in a conventional manner from the E. coli strain, deposited under the provisions of the Budapest Treaty on January 11,
15 2000 at the BCCM-LMBP (Belgian Coordinated Collections of Microorganisms - Laboratorium voor Moleculaire Biologie - Plasmidencollectie, University of Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium) under accession number LMBP 4009, or more preferably, they can be isolated from the supernatant of a Bacillus strain, preferably a crystal-minus Bacillus thuringiensis strain, transformed with a plasmid containing the
20 approximately 7 kb XbaI-EcoRI fragment of the plasmid pUCIB120/ISP as deposited under accession number LMBP 4009. The ISP proteins can be used to prepare specific monoclonal or polyclonal antibodies in a conventional manner (Höfte et al., 1988, Appl. Environm. Microbiol. 54, 2010). The ISP proteins can be treated with a protease such as cysteine proteases, to obtain the protease-resistant fragments of the ISP proteins.

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The DNA sequences encoding the ISP proteins can be isolated in a conventional manner from the deposited strain or can be synthesized based on the encoded amino acid sequence.

5 DNA sequences encoding other ISP proteins in accordance with this invention can be identified by digesting total DNA from isolated bacterial strains with restriction enzymes; size fractionating the DNA fragments, so produced, into DNA fractions of 5 to 10 Kb, preferably 7 to 10 Kb; ligating these fractions to cloning vectors; screening the E. coli, transformed with the cloning vectors, with a DNA probe that was constructed from a region of known ISP
10 protein genes or with a DNA probe based on specific PCR fragments generated from isp DNA using primers corresponding to certain regions within known ISP protein genes. Such "other ISP proteins" isolated in accordance with this invention, like the ISP proteins of this invention, are characterized by any or all, preferably all, of the following characteristics: a) their significant insecticidal activity to larvae of the Southern corn rootworm, Diabrotica undecimpunctata, and to larvae of the Colorado potato beetle, Leptinotarsa decemlineata,
15 upon ingestion of such other ISP protein with an ISP-complimentary protein, preferably the mature ISP1A or ISP2A protein of this invention; b) their lack of significant insecticidal activity to larvae of lepidopteran insects, preferably Ostrinia nubilalis, upon ingestion of such other ISP protein with an ISP-complimentary protein, preferably the mature ISP1A or ISP2A
20 proteins of this invention; c) their apparent molecular weight of about 100 kD or about 45 kD of the mature forms (without signal peptide), d) their occurrence in the supernatant of a bacterial strain culture which is not Bacillus thuringiensis or Bacillus cereus, and e) by their lack of significant toxicity to corn rootworms, preferably Diabrotica virgifera, upon ingestion in the absence of an ISP complimentary protein.

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Of course, any other DNA sequence differing in its codon usage but encoding the same protein or a similar protein with substantially the same insecticidal activity, can be constructed, depending on the particular purpose. It has been described in some prokaryotic and eucaryotic expression systems that changing the codon usage to that of the host cell is desired for gene expression in foreign hosts (Bennetzen & Hall, 1982, J. Biol. Chem. 257, 3026; Itakura, 1977, Science 198, 1056-1063). Codon usage tables are available in the literature (Wada et al., 1990, Nucl. Acids Res. 18, 2367-1411; Murray et al., 1989, Nucleic Acids Research 17, 477-498) and in the major DNA sequence databases. Accordingly, synthetic DNA sequences can be constructed so that the same or substantially the same proteins are produced. It is evident that several DNA sequences can be devised once the amino acid sequence of the ISP proteins of this invention is known. Such other DNA sequences include synthetic or semi-synthetic DNA sequences that have been changed in order to inactivate certain sites in the gene, e.g. by selectively inactivating certain cryptic regulatory or processing elements present in the native sequence, or by adapting the overall codon usage to that of a more related host organism, preferably that of the host organism in which expression is desired. Synthetic DNA sequences could also be made following the procedures described in EP 0 385 962, EP 0 618 967, or EP 0 682 115.

Small modifications to a DNA sequence such as described above can be routinely made by PCR-mediated mutagenesis (Ho et al., 1989, Gene 77, 51-59; White et al., 1989, Trends in Genet. 5, 185-189). New synthetic or semi-synthetic genes can be made by automated DNA synthesis and ligation of the resulting DNA fragments.

To prevent or delay the development of resistance by coleópteran insects, particularly corn rootworm, cotton boll weevil or Colorado potato beetle, preferably Leptinotarsa decemlineata, Diabrotica barberi, Diabrotica undecimpunctata, Anthonomus grandis or

Diabrotica virgifera, to transgenic hosts, particularly plants, expressing ISP proteins of this invention or their equivalents, it is preferred to also express in the same host, preferably a transgenic plant, another protein or another protein complex, which has a different mode of action, and a high toxicity to the same insect targeted by the first toxin or toxin complex when produced in a transgenic host, preferably a plant. Suitable candidates to be combined with the ISP1A and ISP2A of the invention include the mature VIP1Aa protein when combined with the mature VIP2Aa or VIP2Ab protein of PCT publication WO 96/10083 in case these VIP proteins have a different mode of action compared to the ISP proteins; the corn rootworm toxins of *Photorhabdus* or *Xenorhabdus* spp., e.g., the insecticidal proteins of *Photorhabdus luminescens* W-14 (Guo et al., 1999, J. Biol. Chem. 274, 9836-9842); the CryET70 protein of WO 00/26378; the insecticidal proteins produced by Bt strains PS80JJ1, PS149B1 and PS167H2 as described in WO 97/40162, particularly the about 14 kD and about 44 kD proteins of Bt strain PS149B1; the Cry3Bb protein of US patent 6,023,013; protease inhibitors such as the N2 and R1 cysteine proteinase inhibitors of soybean (Zhao et al., 1996, Plant Physiol. 111, 1299-1306) or oryzastatine such as rice cystatin (Genbank entry S49967), corn cystatin (Genbank entries D38130, D10622, D63342) such as the corn cystatin expressed in plants as described by Irie et al. (1996, Plant Mol. Biol. 30, 149-157). Also included herein are all equivalents and variants, such as truncated proteins retaining insecticidal activity, of any of the above proteins.

Such combined expression can be achieved by transformation of a plant already transformed to express a corn rootworm toxic protein or protein complex, or by crossing plants transformed to express different corn rootworm toxic proteins. Alternatively, expression of the ISP proteins of the invention can be induced in roots upon feeding of corn rootworm larvae on root tissue, e.g., by using a wound-induced promoter region, preferably a wound-induced root-preferred promoter region.

WO 01/87931

The 5 to 10, preferably 7 to 10, Kb fragments, prepared from total DNA of the isp genes of the invention, can be ligated in suitable expression vectors and transformed in E. coli, and the clones can then be screened by conventional colony immunoprobings methods (French et al., 1986, Anal.Biochem. 156, 417-423) for expression of the toxin with monoclonal or polyclonal antibodies raised against the ISP proteins. Also, the 5 to 10 Kb fragments; prepared from total DNA of the bacterial strains of the invention, can be ligated in suitable Bt shuttle vectors (Lereclus et al., 1992, Bio/Technology 10, 418) and transformed in a crystal minus Bt-mutant. The clones are then screened for production of ISP proteins (by SDS-PAGE, Western blot and/or insect assay).

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The genes encoding the ISP proteins of this invention can be sequenced in a conventional manner (Maxam and Gilbert, 1980, Methods in Enzymol. 65, 499-560; Sanger, 1977, Proc. Natl. Acad. Sci. USA 74, 5463-5467) to obtain the DNA sequence. Sequence comparisons indicated that the genes are different from previously described genes encoding proteins secreted during the vegetative growth phase of Bacillus or other bacterial species and Bacillus thuringiensis crystal proteins with activity against Coleoptera (Crickmore, et al., 1998, Microbiology and Molecular Biology Reviews Vol 62: 807-813; WO 98/44137, WO 94/21795, WO 96/10083, WO 00/09697, WO 99/57282, and WO 97/46105).

20 In order to express all or an insecticidally effective part of the DNA sequence encoding an ISP protein of this invention in E. coli, in other bacterial strains or in plants, suitable restriction sites can be introduced, flanking the DNA sequence. This can be done by site-directed mutagenesis, using well-known procedures (e.g., Stanssens et al., 1989, Nucleic Acids Research 12, 4441-4454; White et al., 1989, supra). For expression in plants, the isp DNA of the invention, or its equivalent, is usually contained in a chimeric gene, flanked by 5' and 3' regulatory sequences including a plant-expressible promoter region and 3'

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transcription termination and polyadenylation sequence active in plant cells. In order to obtain improved expression in plants, the codon usage of the isp DNA or its equivalent of this invention can be modified to form an equivalent, modified or artificial gene or gene part, or the isp DNA or insecticidally-effective parts thereof can be inserted in the chloroplast genome and expressed there using a chloroplast-active promoter (e.g., Mc Bride et al., 1995, Bio/Technology 13, 362). For obtaining enhanced expression in monocot plants such as corn, a monocot intron also can be added to the chimeric gene, and the isp DNA sequence or its insecticidal part can be further changed in a translationally neutral manner, to modify possibly inhibiting DNA sequences present in the gene part by means of site-directed intron insertion and/or by introducing changes to the codon usage, e.g., adapting the codon usage to that most preferred by the specific plant (Murray et al., 1989, supra) without changing significantly the encoded amino acid sequence.

The insecticidally effective isp DNA or its equivalent, preferably the isp chimeric gene, encoding an ISP protein, can be stably inserted in a conventional manner into the nuclear genome of a single plant cell, and the so-transformed plant cell can be used in a conventional manner to produce a transformed plant that is insect-resistant. In this regard, a disarmed Ti-plasmid, containing the insecticidally effective isp gene part, in Agrobacterium tumefaciens can be used to transform the plant cell, and thereafter, a transformed plant can be regenerated from the transformed plant cell using the procedures described, for example, in EP 0 116 718, EP 0 270 822, PCT publication WO 84/02913 and published European Patent application ("EP") 0 242 246 and in Gould et al. (1991, Plant Physiol. 95, 426-434). Preferred Ti-plasmid vectors each contain the insecticidally effective isp chimeric gene sequence between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for

example in EP 0 233 247), pollen mediated transformation (as described, for example in EP 0 270 356, PCT publication WO 85/01856, and US Patent 4,684,611), plant RNA virus-mediated transformation (as described, for example in EP 0 067 553 and US Patent 4,407,956), liposome-mediated transformation (as described, for example in US Patent 4,536,475), and other methods such as the methods for transforming certain lines of corn (Fromm et al., 1990, Bio/Technology 8, 833-839; Gordon-Kamm et al., 1990, The Plant Cell 2, 603-618, US Patent 5,767,367) and rice (Shimamoto et al., 1989, Nature 338, 274-276; Datta et al., 1990, Bio/Technology 8, 736-740) and the recently described method for transforming monocots generally (PCT publication WO 92/09696).

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Different conventional procedures can be followed to obtain combined expression of two ISP proteins in transgenic plants as summarized below:

I. Chimeric gene constructs whereby two ISP DNA sequences and a marker gene are transferred to the plant genome as a single piece of DNA and lead to the insertion in a single locus in the genome.

15 Ia. The genes can be engineered in different transcriptional units each under control of a distinct promoter.

To express two ISP proteins and a marker protein as separate transcriptional units, several promoter fragments directing expression in plant cells can be used as described above. All combinations of the promoters mentioned above in the chimaeric constructs for one ISP gene are possible. The ISP coding region in each chimeric gene of this invention can be the intact isp gene or preferably an insecticidally-effective part of the intact isp gene. The individual chimeric genes are cloned in the same plasmid vector according to standard procedures (e.g., EP 0 193 259).

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Ib. Two genes (e.g., either an isp and a marker gene or two isp genes) or more can be combined in the same transcriptional unit.

To express two isp genes in the same transcriptional unit, the following cases can be distinguished:

In a first case, hybrid genes in which the coding region of one gene is fused in frame
5 with the coding region of another gene can be placed under the control of a single promoter. Fusions can be made between either an isp and a marker gene or between two isp genes.

Also, between each gene encoding an ISP or an insecticidally-effective fragment thereof, a gene fragment encoding a protease (e.g., trypsin, cysteine or serine protease)-
10 sensitive protein part could be included, such as a gene fragment encoding a part of the ISPs which is removed or cleaved upon activation by the midgut enzymes of the target insect species. Alternatively, between each gene encoding an ISP or an insecticidally-effective fragment thereof, a gene fragment can be included encoding a peptide of about 16-
20 amino acids which has the capability to mediate cleavage at its own C-terminus by an
15 enzyme-independent reaction (Halpin et al., 1999, Plant J. 17, 453-459, US Patent 5,846,767), or a gene fragment encoding a linker peptide sequence which allows the production of a recombinant cell with significant toxicity to the target insects.

In a second case, the coding regions of the two respective isp genes can be combined in
20 dicistronic units placed under the control of a promoter. The coding regions of the two isp genes are placed after each other with an intergenic sequence of defined length. A single messenger RNA molecule is generated, leading to the translation into two separate gene products. Based on a modified scanning model (Kozak, 1987, Mol. Cell. Biol. 7, 3438-3445), the concept of reinitiation of translation has been accepted provided that a termination codon
25 in frame with the upstream ATG precedes the downstream ATG. Experimental data also

demonstrated that the plant translational machinery is able to synthesize several polypeptides from a polycistronic mRNA (Angenon et al., 1989, Mol. Cell Biol. 9, 5676-5684).

Based on the mechanism of internal initiation of translation (Jackson and Kaminski, 1995, RNA 1, 985-1000) initiation of translation of the second gene occurs by binding of the 43S pre-initiation complex to a specific intergenic sequence (internal ribosome entry sequence; IRES). Experimental data also demonstrated that the plant translational machinery is able to synthesize several polypeptides from a polycistronic mRNA containing intergenic IRES-elements (Hefferon et al., 1997, J Gen Virol 78, 3051-3059; Skulachev et al., 1999, Virology 263, 139-154; PCT patent publication WO 98/54342).

II. A chimeric construct with one isp gene that is transferred to the genome of a plant already transformed with one isp gene :

Several genes can be introduced into a plant cell during sequential transformation steps (retransformation), provided that an alternative system to select transformants is available for the second round of transformation, or provided that the selectable marker gene is excised from the plant genome using DNA recombination technology (e.g., published PCT applications WO 94/17176 and WO 91/09957). This retransformation leads to the combined expression of isp genes which are introduced at multiple loci in the genome. Preferably, two different selectable marker genes are used in the two consecutive transformation steps. The first marker is used for selection of transformed cells in the first transformation, while the second marker is used for selection of transformants in the second round of transformation. Sequential transformation steps using kanamycin and hygromycin have been described, for example by Sandler et al. (1988, Plant Mol. Biol. 11, 301-310) and Delauney et al. (1988, Proc. Natl. Acad. Sci. U.S.A. 85, 4300-4304).

III. Chimeric constructs with isp genes, that are separately transferred to the nuclear genome of separate plants in independent transformation events and are subsequently combined in a single plant genome through crosses.

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The first plant should be a plant transformed with a first isp gene or an F1 plant derived thereof through selfing (preferably an F1 plant which is homozygous for the isp gene). The second plant should be a plant transformed with a second isp gene or an F1 plant derived thereof through selfing (preferably an F1 plant which is homozygous for the second isp gene). Selection methods can be applied to the plants obtained from this cross in order to select those plants having the two isp genes present in their genome (e.g., Southern blotting) and expressing the two ISPs (e.g., separate ELISA detection of the immunologically different ISPs). This is a useful strategy to produce hybrid varieties from two parental lines, each transformed with a different isp gene, as well as to produce inbred lines containing two different isp genes through crossing of two independent transformants (or their F1 selfed offspring) from the same inbred line.

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IV. Chimeric constructs with one or more isp genes separately transferred to the genome of a single plant in the same transformation experiment leading to the insertion of the respective chimeric genes at the same or at multiple loci.

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Cotransformation involves the simultaneous transformation of a plant with two different expression vectors, one containing a first isp gene, the second containing a second isp gene. Along with each isp gene, a different marker gene can be used, and selection can be made with the two markers simultaneously. Alternatively, a single marker can be used, and a sufficiently large number of selected plants can be screened in order to find those plants

having the two isp genes (e.g., by Southern blotting) and expressing the two proteins (e.g., by means of ELISA). Cotransformation with more than one T-DNA can be accomplished by using simultaneously two different strains of Agrobacterium, each with a different Ti-plasmid (Depicker et al., 1985, Mol. Gen. Genet. 201, 477-484) or with one strain of Agrobacterium containing two T-DNAs on separate plasmids (de Framond et al., 1986, Mol. Gen. Genet. 202, 125-131). Direct gene transfer, using a mixture of two plasmids or fragments thereof, can also be employed to cotransform plant cells with a selectable and a non-selectable gene (Schocher et al., 1986, Bio/technology 4, 1093-1096).

10 The resulting transformed plant can be used in a conventional plant breeding scheme to produce more transformed plants with the same characteristics or to introduce the insecticidally effective isp gene in other varieties of the same or related plant species. Seeds, which are obtained from the transformed plants, contain the insecticidally effective isp gene as a stable genomic insert. Cells of the transformed plant can be cultured in a conventional manner to produce the insecticidally effective portion of the ISP protein which can be recovered for use in conventional insecticide compositions against insects. Of course, the above possibilities of combined production of ISP proteins in plants is as well applicable to active fragments of the ISP proteins or the ISP equivalents of this invention.

The insecticidally effective isp gene is inserted in a plant cell genome so that the inserted gene is downstream (i.e., 3') of, and operably linked to, a promoter which can direct the expression of the gene part in the plant cell. This is preferably accomplished by inserting the isp chimeric gene in the plant cell genome, particularly in the nuclear or chloroplast genome. Preferred promoters include: the strong constitutive 35S promoters (the "35S promoters") of the cauliflower mosaic virus (CaMV) of isolates CM 1841 (Gardner et al., 1981, Nucl. Acids Res. 9, 2871-2887), CabbB-S (Franck et al., 1980, Cell 21, 285-294) and CabbB-JI (Hull and
25 Howell, 1987, Virology 86, 482-493); promoters from the ubiquitin family (e.g., the maize

ubiquitin promoter of Christensen et al., 1992, Plant Mol. Biol. 18, 675-689; see also Cornejo et al., 1993, Plant Mol. Biol. 23, 567-581), the *gos2* promoter (de Pater et al., 1992, Plant J. 2, 837-844), the *emu* promoter (Last et al., 1990, Theor. Appl. Genet. 81, 581-588), rice actin promoters such as the promoter described by Zhang et al. (1991, The Plant Cell 3, 1155-1165); and the TR1' promoter and the TR2' promoter (the "TR1' promoter" and "TR2' promoter", respectively) which drive the expression of the 1' and 2' genes, respectively, of the T-DNA (Velten et al., 1984, EMBO J 3, 2723-2730). Alternatively, a promoter can be utilized which is not constitutive but rather is specific for one or more tissues or organs of the plant (e.g., leaves and/or roots) whereby the inserted *isp* gene is expressed only in cells of the specific tissue(s) or organ(s). In a preferred embodiment of the invention, the insecticidally effective *isp* gene is selectively expressed in the roots of a plant, preferably corn, by placing the insecticidally effective gene part under the control of a root-preferred promoter (i.e., a promoter which is most active in root tissue, preferably a promoter with no or little transcription in non-root tissue such as pollen, leaves and stem, more particularly a promoter only resulting in detectable transcription in root tissue). Root-preferred promoters need not be exclusively active in root tissue. Root-preferred promoters in accordance with this invention include but are not limited to: promoters located immediately upstream of a DNA sequence corresponding to a root-specific cDNA, preferably from corn; the promoter of US patent 5,837,876, published PCT patent applications WO 00/29594, WO 00/73474, WO 01/00833, or US Patent 6,008,436; the ZRP2 promoter of US Patent 5,633,363 and Held et al. (1997, Plant Mol. Biol. 35, 367-375, Genbank accession number U38790); the promoter of US Patent 5,817,502; the promoter described by de Framond (1991, FEBS 290: 103-106; EP 0 452 269), the root-specific promoter of the peroxidase gene POX1 from wheat (Hertig et al., 1991, Plant Molec. Biol. 16, 171-174), the promoter of US patent 5,837,848, the promoter of PCT publication WO 015662, the promoter of Goddemeier et al. (1998, Plant Mol. Biol. 36, 799-802). Other root-specific or root-preferred promoters which may be useful in this invention

include the promoters described by Hirel et al., 1992, Plant Mol. Biol. 20, 207; Keller and Baumgartner, 1991, The Plant Cell 3, 1051-1061; Sanger et al., 1990, Plant Mol. Biol. 14, 483-443; Miao et al., 1991, The Plant Cell 3, 11-22; Bogusz et al., 1990, The Plant Cell, 2, 633-641; Leach and Aoyagi, 1991, Plant Science 79, 69-76; Teeri et al., 1989, EMBO Journal 8, 343-350, all of which are incorporated herein by reference.

Expression in leaves of a plant can be achieved by using the promoter of the ribulose-1,5-bisphosphate carboxylase small subunit gene of the plant itself or of another plant such as pea as disclosed in US Patent 5,254,799. Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical factors). Also, for corn plants, particularly at the time adult corn rootworms are present in corn rootworm fields, expression in pollen is preferred, by using promoter regions resulting in expression in corn pollen, e.g. the promoters described in published PCT applications WO 93/25695 and WO 01/12799.

The insecticidally effective isp gene is inserted in the plant genome so that the inserted gene part is upstream (i.e., 5') of suitable 3' end transcription regulation signals (i.e., transcript formation and polyadenylation signals).

This is preferably accomplished by inserting the isp chimeric gene in the plant cell genome. The choice of the 3' regulatory sequence in the chimeric gene of the invention is not critical. In some cases good expression can be obtained when no such region is present in the chimeric gene, since a DNA sequence at the insertion site can act as a 3' regulatory sequence. Preferred polyadenylation and transcript formation signals include those of the CaMV 35S (Mogen et al., 1990, The Plant Cell 2, 1261-1272), the octopine synthase gene (Gielen et al., 1984, EMBO J 3, 835-845), the nopaline synthase gene (Depicker et al., 1982,

J. Mol. Appl. Genet. 1, p. 561), and the T-DNA gene 7 (Velten and Schell, 1985, Nucleic Acids Research 13, 6981-6998), which act as 3'-untranslated DNA sequences in transformed plant cells.

- 5 The insecticidally effective isp gene can optionally be inserted in the plant genome as a hybrid gene (US Patent 5,254,799; Vaeck et al., 1987, Nature 327, 33-37) under the control of the same promoter as a selectable marker gene, such as the neo gene (EP 0 242 236) encoding kanamycin resistance, so that the plant expresses a fusion protein.
- 10 All or part of the isp gene, can also be used to transform other bacteria, such as a B. thuringiensis strain which has insecticidal activity against Lepidoptera or Coleoptera, or root-colonizing bacteria, e.g. root-colonizing Pseudomonas putida (Vilchez et al., J. Bacteriol. 182, 91-99). Thereby, a transformed bacterial strain can be produced which is useful for combatting insects and which can affect a wide spectrum of lepidopteran and coleopteran
- 15 insect pests. Transformation of bacteria, such as bacteria of the genus Agrobacterium, Bacillus or Escherichia, with all or part of the isp gene of this invention or its equivalent, incorporated in a suitable cloning vehicle, can be carried out in a conventional manner, such as heat shock transformation (Bergmans et al., 1981, J. Bacteriol 146, 564-570) or conventional electroporation techniques as described in Mahillon et al. (1989, FEMS
- 20 Microbiol. Letters 60, 205-210) and in PCT Patent publication WO 90/06999.

Transformed bacterial strains, such as Bacillus species strains, preferably Bacillus thuringiensis strains, containing the isp gene of this invention can be fermented by conventional methods (Dulmage, 1981, "Production of Bacteria for Biological Control of

25 Insects" in Biological Control in Crop Production, Ed. Paparizas, D.C., Osmun Publishers, Totowa, N.J., USA, pp. 129-141; Bernhard and Utz, 1993, "Production of Bacillus

thuringiensis insecticides for experimental and commercial uses", In Bacillus thuringiensis, An Environmental Biopesticide: Theory and Practice, pp.255-267, eds. Entwistle, P.F., Cory, J.S., Bailey, M.J. and Higgs, S., John Wiley and Sons, New York) to provide high yields of cells.

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An insecticidal, particularly anti-coleopteran, preferably anti-corn rootworm composition of this invention can be formulated in a conventional manner using the microorganisms transformed with the isp gene, or preferably their respective ISP proteins or insecticidally effective portions thereof as an active ingredient, together with suitable carriers, diluents, emulsifiers and/or dispersants (e.g., as described by Bernhard and Utz, 1993, supra). This insecticide composition can be formulated as a wettable powder, pellets, granules or dust or as a liquid formulation with aqueous or non-aqueous solvents as a foam, gel, suspension, concentrate, etc. Known microorganisms include cells of Pseudomonas or other bacteria which serve to encapsulate the proteins in a stable environment prior to application to the insects. Also included in the invention is a product comprising the ISP1A and ISP2A protein of the invention as a combined preparation for simultaneous, separate or sequential use to protect corn plants against corn rootworms, particularly such product is an insecticidal composition or a transgenic corn plant.

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A method for controlling insects, particularly Coleoptera, in accordance with this invention can comprise applying (e.g., spraying), to a locus (area) to be protected, an insecticidal amount of the ISP proteins or host cells transformed with the isp gene of this invention. The locus to be protected can include, for example, the habitat of the insect pests or growing vegetation or an area where vegetation is to be grown.

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To obtain the ISP protein, cells of the recombinant hosts expressing the ISP protein can be grown in a conventional manner on a suitable culture medium and the protein can then be obtained from the medium using conventional means. The ISP protein can then be separated and purified by standard techniques such as chromatography, extraction, electrophoresis, or the like. The protease-resistant toxin form can then be obtained by protease, e.g. cysteine or serine protease, digestion of the protein.

Bio-assays for testing the insecticidal efficacy are well known in the art. A method for corn rootworm testing is described in Marrone et al. (1985, J. Econ. Entomol. 78, 290-293), but many other methods are available to test insects on artificial diet or on transgenic plants, e.g. US Patent 5,990,383. In a suitable bio-assay the proper controls are included to check for background mortality.

The following Examples illustrate the invention, and are not provided to limit the invention or the protection sought. Many variants or equivalents of these Examples can be made or designed by a person of ordinary skill in the art without departing from the teachings of the invention and the common knowledge. The sequence listing referred to in this application (which forms part of this application and is attached thereto), is as follows:

Sequence Listing:

- SEQ ID No. 1 - amino acid and DNA sequence of ISP1A protein and DNA
- SEQ ID No. 2 - amino acid sequence of ISP1A protein.
- SEQ ID No. 3 - amino acid and DNA sequence of ISP2A protein and DNA.
- SEQ ID No. 4 - amino acid sequence ISP2A protein.
- SEQ ID No. 5 - DNA sequence of the short zrp2 promoter fragment (n= any nucleotide)
- SEQ ID No. 6 - DNA sequence of the long zrp2 promoter fragment (n=any nucleotide)

WO 01/87931

SEQ ID No. 7 - DNA sequence encoding ISP2A-1 protein

SEQ ID No. 8 - amino acid sequence of ISP2A-1 protein

SEQ ID No. 9 - DNA sequence encoding ISP1A-1 protein fragment

SEQ ID No. 10 - amino acid sequence of ISP1A-1 protein

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Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA are carried out by the standard procedures described in Sambrook et al., Molecular Cloning - A Laboratory Manual, Second Ed., Cold Spring Harbor Laboratory Press, NY (1989), and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular biology work are described in Plant Molecular Biology Labfax (1993) by R.R.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK). Procedures for PCR technology can be found in "PCR protocols: a guide to methods and applications", Edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Academic Press, Inc., 1990).

EXAMPLES

Example 1: Characterization of the IB120-A strain

20

A bacterial strain, named herein the IB120-A strain, was found in a field in the state of Morelos, Mexico.

The strain was grown overnight on LB agar plates (LB medium with 1.5 % agar added; LB medium: 10 g/l trypton, 10 g/l NaCl, 5 g/l yeast extract, pH 7.3) at 28°C. For small scale cultures, 20 ml TB medium (Terrific Broth: 12 g/l tryptone, 24 g/l yeast extract, 3.8 g/l KH_2PO_4 , 12.5 g/l K_2HPO_4 , 5 ml/l glycerol, pH 7.1) was inoculated and grown for 65 hours at 28°C on a

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rotating platform having about 70 rotations per minute. After 65 hours, a protease inhibitor mixture was added to the culture. This cocktail has the following ingredients (volumes given are those required to add to one 20 ml culture): 200µl PMSF (100mM), 200µl of a mixture of benzamidine.HCl (100mM) and epsilon-amino-n-caproic acid (500 mM), 400µl EGTA (0.5M), 5 40µl antipain (0.5mg/ml) / leupeptin (0.5mg/ml) and 20µl beta-mercapto ethanol (14M). The culture medium to which the protease inhibitor mixture had been added, was then centrifuged for 20 minutes at 3000 rpm. In some cases, the supernatant was concentrated about 4 times using Centriprep YM-10 Centrifugal Filter Devices (Millipore Cat. No. 4305). For long term storage, a loop of sporulated cells was added to 0.5ml of 25% or 50% glycerol and after 10 vortexing, stored at -70°C. Sporulated cells were obtained by growth of the strain on LB agar plates until sporulation (as apparent under the light microscope).

After cultivating on LB agar plates of single cell colonies, microscopical analysis of the IB120-A strain culture showed the presence of rod-shaped motile single vegetative cells and swollen 15 sporangia containing an ovale spore. No parasporal crystals were detected in cultures of the IB120-A strain.

Supernatant of the IB120-A strain showed strong toxicity to Diabrotica virgifera larvae (hereinafter termed "Dv") in surface contamination assays on artificial diet (diet as described by 20 Sutter et al. (1971, J. Econ. Entomol. 64, 65-67)). The assays were done at 24 °C (+/- 1°C) in 24 multiwell Costar plates at 50 microliter toxin solution per well (2cm²), 6 wells were analyzed with 4 L1 (first instar) larvae per well for each sample (scoring after 7 days).

Screening of supernatant harvested at 7, 24, 30, 48 and 120 hours after initiation of the culture 25 showed the strongest toxicity to Dv at 48 hours, with no significant toxicity found at 7 hours. At

48 hours, mortality in the 50-60 % range was still found when the supernatant (at total protein concentration of 214 microgr/ml) was diluted at 1/1024.

5 Loss of activity of IB120-A supernatant harvested 48, 65 and 144 hours after culture initiation (in dilutions of 1/1 to 1/8) upon heat treatment and retention of activity after ammonium sulphate precipitation indicated that the toxic compound is likely a protein.

Preliminary characterization of the IB120-A strain by PCR primers for gyrase B genes (Yamada et al., 1999, Appl. Environm. Microbiol. 65, 1483-1490) suggested that it was not a Bacillus strain of the subspecies thuringiensis, anthracis or cereus. Also, no amplification products were obtained using PCR primers specifically characterizing the 16S RNA sequences of the genus Paenibacillus which are also recognizing the species Bacillus lentimorbus and Bacillus popilliae (Pettersson et al., 1999, Int J Syst Bacteriol. 49(2), 531-540). Growth in NB (nutrient broth: 3 g/l bacto beef extract, 5 g/l bacto peptone, pH 6.8) medium indicated that the new strain was not a Bacillus larvae species. Thus, this IB120-A strain seems to be different from previously isolated Bacillus strains known to produce secreted insecticidal proteins which are not contained in crystals.

Based on the rod-like shape and the aerobic growth, the strain could be a Bacillus species strain. With a set of general and specific primers for cry3 Bacillus thuringiensis genes, no amplification products were found when analyzing strain IB120-A.

Detailed analysis of the IB 120-A strain using standard microbial identification techniques, including fatty acid analysis and API 50CHB tests combined with API 20E tests, showed that this strain is a Brevibacillus laterosporus species strain.

Example 2. Isolation and characterization of isp1 and isp2 DNAs/proteins:

In order to isolate the genes responsible for the toxicity of IB120-A, total DNA from this strain was prepared and partially digested with Sau3A. The digested DNA was size fractionated on a sucrose gradient and fragments ranging from 7 kb to 10 kb were ligated to the BamH1-digested and TsAP (thermosensitive alkaline phosphatase)- treated cloning vector pUC19 (Yannisch-Perron et al, 1985, Gene 33, 103-119.). The ligation mixture was electroporated in E. coli XL1-Blue cells. Transformants were plated on LB-triacillin plates containing Xgal and IPTG and white colonies were selected to be used in filter hybridization experiments. Recombinant E. coli clones containing the vector were then screened with a DIG labeled probe which was prepared as follow. First, a PCR was performed using as template cells from strain IB120-A. The resulting amplification product was gel-purified and used as template in a secondary PCR reaction using DIG- labeled dNTPs and the same primers as in the first PCR reaction. An appropriate amount of this amplification product was used in hybridization reactions.

15

Following the identification of a positive colony containing a plasmid harboring the full length isp genes, the sequence of these genes was determined using the dye terminator labeling method and a Perkin Elmer ABI Prism-377 DNA sequencer. The sequences of the 2 open reading frames found in the cloned DNA fragment of a positive colony are shown in SEQ ID No. 1 and 3. These DNA sequences were found to be organized in an operon and encode novel proteins, ISP1A (SEQ ID No. 2) and ISP2A (SEQ ID No. 4) which have been found to be the causal agents of the high insecticidal activity observed. A positive colony containing the pUC-derived plasmid with the genes responsible for toxicity (in plasmid pUCIB120/ISP) has been deposited under the provisions of the Budapest treaty in E. coli XL1Blue as LMBP 4009 on January 11, 2000.

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WO 01/87931

A plasmid preparation was made from the positive colony and this plasmid was cut using XbaI and EcoRI (resulting in an about 7 Kb fragment) and ligated in the shuttle vector pSL40 which had been cut using the same restriction enzymes. This yielded plasmid pSLIB120/ISP. The plasmid pSLIB120/ISP was then transferred into a crystal- minus B. thuringiensis strain.

5 Supernatant from this recombinant Bt strain was obtained as follows.

The strain was grown overnight on LB agar plates containing erythromycin (20 µg/ml) at 28°C. For small scale cultures, 20 ml TB medium containing erythromycin (20 µg/ml) was inoculated and grown for 65 hours at 28°C on a rotating platform having about 70 rotations per minute. 10 After 65 hours, a protease inhibitor mixture was added to the culture. This cocktail has the following ingredients (volumes given are those required to add to one 20 ml culture): 200µl PMSF (100mM), 200µl of a mixture of benzamidine.HCl (100mM) / epsilon-amino-n-caproic acid (500 mM), 400µl EGTA (0.5M), 40µl antipain (0.5 mg/ml) / leupeptin (0.5 mg/ml) and 20µl beta-mercapto ethanol (14M). The culture medium to which the protease inhibitor mixture had 15 been added, was then centrifuged for 20 minutes at 3000 rpm. In some cases, the supernatant was concentrated about 4 times using centriprep YM-10 Centrifugal Filter Devices (Millipore, Cat. No. 4305).

Insecticidal activity of the supernatant at 48 hours after culture initiation of the recombinant Bt strain containing the pSLIB120/ISP plasmid using the Dv surface contamination assay 20 described above, showed that the supernatant still had significant mortality in the 60 % range at a 1/1024 dilution, while control mortality (the controls included supernatant of the untransformed Bt strain) was 0 %. This shows that the isolated DNA encodes an insecticidal ingredient of the IB120-A strain, and upon transfer to another bacterium is also expressed and secreted in the 25 culture medium. Analysis of toxicity of another independent Bt-crystal-minus strain transformed

with the pSLIB120/ISP plasmid confirmed the high insecticidal activity of the supernatants compared to that of the untransformed control.

The LC50 value of the supernatant of the Bt strain expressing the two ISP proteins was found to be 3.5 ng/cm² after 4 days using the above-described surface contamination assay with Dv larvae (based on total supernatant protein concentration). Detailed analysis of the toxicity of supernatant produced by the recombinant Bt strain expressing the ISP1A and ISP2A proteins to selected coleopteran insects is reported in Table 1 below. The ISP proteins of the invention were found to have significant insecticidal activity to Western, Northern and Southern corn rootworm larvae, and also to larvae of the Colorado potato beetle and the cotton boll weevil.

10

The mature ISP1 and ISP2 proteins were purified to apparent homogeneity by ammonium sulphate precipitation followed by passage over different chromatographical columns. The chromatographical analysis suggest that the ISP1A and ISP2A proteins are present in the supernatant in equimolar ratio. The ISP1A protein was found to be of rather hydrophobic nature, while ISP2A was of rather hydrophilic nature. Amino-terminal sequence determination of the mature ISP1A and ISP2A proteins produced in the recombinant crystal-minus Bt strain showed that amino acid position 38 in SEQ ID No. 2 for ISP1A and amino acid position 51 in SEQ ID No. 4 for ISP2A are the N-terminal amino acids of the active proteins present in supernatant of such transformed Bt strain. The N-terminus for ISP1A was found to be IATTTQASKD, that for ISP2A was found to be LVKTTNNTED, which matches fully with the amino acid sequence of the DNA sequences isolated.

20

The apparent molecular weight of the pure mature ISP1A protein was determined to be about 100 kD in 8% SDS-PAGE gel electrophoresis, that of the pure mature ISP2A protein was determined to be about 45 kD protein in 10 % SDS-PAGE gel electrophoresis, using molecular weight markers of 37, 50, 75, 100, and 150 kD.

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The activity of the mature ISP1A and ISP2A proteins as produced by the recombinant strain was evaluated against several insects. The results against selected coleopteran insects are shown in Table 1 below.

5 **Table 1: Coleopteran activity of ISP1A-ISP2A :**

Insect	Stage	LC50 (µg/ml) (95% CL)	LC90 (µg/ml) (95% CL)
Dv	L1	0.437	1.689
		(0.321-0.563)	(1.250-2.597)
	L2	3.84	117.4
		(-)	(-)
	L3	21.674*	531.76
		(5.012-100.432)	(110.688-86864)
Db	L1	0.213	0.890
		(0.116-0.338)	(0.542-2.006)
Du	L1	4.91	30.06
		(1.65-13.26)	(11.52-329.72)
Ld	L1+2d	0.037	1.068
		(-)	(-)
Ag	L1	207.1	8759.2
		(84.3-654.7)	(1865.8-620175.8)

Legend to Table 1:

Dv: Diabrotica virgifera, Western corn rootworm; Db: Diabrotica barberi, Northern corn rootworm; Du: Diabrotica undecimpunctata howardi, Southern corn rootworm; Ld: Leptinotarsa decemlineata, Colorado potato beetle; Ag: Anthonomus grandis, cotton boll weevil; L1: first larval stage; L2: second larval stage; L3: third larval stage; L1+2d: 2d after egg hatch; *: 90%CL (CL=Confidence Limits, LC50: total supernatant protein concentration when 50% of the insects are killed).

Bioassays used: Dv, Db, Du: surface contamination assay on artificial diet (see above for description) at ratio of 25 µl/cm², scoring after 7 days; Ld: diptest with potato foliage (cut potato foliage dipped in toxin solution, allowed to dry and put in a petri dish (9 cm diam.) with 10 larvae; after 1 day untreated foliage was added to the petridish, scoring was after 5 days); Ag:

artificial diet incorporation test : diet as described by Moore and Whisnant, Handbook of Insect Rearing, Vol. I, Pritah Singh and R.F. Moore; Elsevier, Amsterdam, 1985, p. 217; assay: incorporation of 2 ml toxin solution per 25 ml diet, in 24 multiwell Costar plates (about 1 ml/well), 1 egg per well, 20 wells per sample, scoring after 14 days).

- 5 In the assays reported in Table 1, controls (untreated food, food supplied with supernatant of a non-transformed Bt-crystal-minus strain or with water) did not show any mortality above 20 % for any of the above insects (a variation from 5 to 20 % control mortality was found according to larval stage and insect species (the 20 % value is for the third larval stage of Dv)).
- 10 The LC₅₀ values obtained for Diabrotica virgifera larvae using the bio-assay as described above, when an equimolar combination of ISP1A and ISP2A protein was applied after purification of these proteins from the supernatans of the recombinant Bt strain, were not significantly different from those obtained above.
- 15 Bio-assays of a mixture of the mature ISP1A and ISP2A protein in standard surface contamination assays against selected lepidopteran insects (Ostrinia nubilalis, Heliothis virescens, Helicoverpa zea, Spodoptera frugiperda, Sesamia nonagrioides, Spodoptera littoralis, Helicoverpa armigera, and Manduca sexta) showed that the ISP proteins of this invention do not cause any mortality in these insects above control levels. This evidences the
- 20 coleopteran-specific nature of the proteins of this invention. Preliminary bio-assays with two aphid species also showed that a mixture of the ISP1A and ISP2A proteins did not cause any significant mortality in these insects.

- To determine which fragment of the isp genes are sufficient to encode an ISP protein complex
- 25 toxic to Diabrotica larvae, a series of C-terminally truncated ISP proteins were made by inserting a stopcodon in the ORF of the isp genes at different positions (and thus making

different 3' gene truncations). The stopcodons were inserted at random positions using the Genome Priming System (GPS, New England Biolabs, catalog #7100). Using this system, a 1.7 kb transposon (containing stopcodons in all three reading frames and 2 sequences complementary to specific primers) is inserted randomly but only once in the 'target' DNA. The position of the insertion, and therefore, the truncation can then be estimated by PCR screening using a gene-specific primer and a transposon-specific primer or can be precisely determined by sequencing. A set of constructs with a transposon at different positions in one of the isp genes was then transformed individually in a crystal minus Bt strain and the susceptibility of Western corn rootworm larvae to the supernatant of each recombinant Bt strain was tested using the assay described above.

For truncation of the isp2 gene, a fragment containing the gene was cut out of pSLIB120/ISP using BstBI and PmlI. Following the GPS reaction, this fragment was then ligated into the pSLIB120/ISP vector, cut with the same enzymes. Recombinant E. coli colonies were PCR-screened in order to estimate the position of the transposon insertion site. A set of 10 clones with the transposon at different positions in the second half (3' half) of the isp2 gene was selected. Plasmid DNA of these clones was transformed in a dcm and dam methylase negative E. coli strain GM2163. Plasmid DNA isolated from this strain was then transformed in a crystal-minus Bt strain. Supernatant was prepared and bioassayed, using supernatant from the Bt strain transformed with pSLIB120/ISP as a positive control and supernatant from the crystal-minus Bt strain as a negative control. For truncation of the isp1 gene, a fragment was cut out of pSLIB120/ISP using PmlI and EcoRI. The same methodology was followed as for the truncation of the isp2 gene.

The results of the transposon insertion analysis indicate that truncation of relatively large segments of the isp2A gene at the 3' end abolishes toxicity, indicating that no or only a

relatively short C-terminal truncation is allowed in the ISP2A protein. After sequencing, the smallest C-terminally truncated ISP2A toxin fragment retaining toxicity in this study when tested in combination with the ISP1A protein was found to end at amino acid position 449 in SEQ ID No. 4.

The results of these analyses indicate that a larger truncation of the ISP1A protein at the 3' end is possible: sequence determination showed that the toxic fragment with the largest C-terminal truncation ended at amino acid position 768 in SEQ ID No. 2. Thus, approximately 300 bp of the ISP1A coding region can be deleted at the 3' side without losing toxicity.

The above study also indicates that insecticidal activity is reduced to the background level when no functional ISP1A is produced, confirming the binary nature of the ISP proteins of this invention.

The ISP2A protein from amino acid position 51 to amino acid position 449 in SEQ ID No. 4 and the ISP1A protein from amino acid position 38 to amino acid position 768 in SEQ ID No. 2 are thus useful insecticidal fragments of the ISP proteins.

Furthermore, a chimeric gene containing a DNA fusion of the ISP1A and ISP2A genes was expressed in a crystal-minus Bacillus thuringiensis berliner 1715 strain. A DNA sequence encoding an Arg-Lys-linker (RKRKRK) or a DNA sequence encoding a Gly-linker (GGGGGG) was provided between the open reading frames encoding the ISP2A and the ISP1A proteins (ISP2A still has its N-terminal signal peptide present, while ISP1A lacks its signal peptide), so that a translational fusion protein is produced; i.e. a fusion protein in which the specified linker connects the ISP2A protein (as the N-terminal fusion partner) to the ISP1A protein (as the C-terminal fusion partner). The recombinant Bt strain proved to cause mortality to Diabrotica

virgifera which was significantly higher than that of the untransformed control strain (i.e., the crystal-minus Bt 1715 strain). This shows the possibility of producing the ISP proteins of the invention as a fusion protein in a recombinant host cell by using a single chimeric gene. Obviously, as explained in the description of the invention, multiple different approaches can be used to achieve the production of a fusion protein in the recombinant cell, e.g. cells of a plant.

Example 3: production of ISP proteins in plants:

To obtain optimal expression in plants, the isp1A and isp2A native DNA sequences are modified to produce modified DNA sequences encoding the ISP1A and ISP2A proteins. The optimized DNA sequences encoding the ISP1A-1 and ISP2A-1 proteins with their signal peptides replaced by a methionine and alanine amino acid are shown in SEQ ID No. 9 and 7, respectively.

These coding regions are inserted in a chimeric gene operably linked to suitable regulatory sequences, e.g., a promoter based on the short or long zrp2 promoter sequences of SEQ ID No. 5 or 6 using standard techniques, and appropriate 3' transcription termination and polyadenylation sequences. In some constructs, the above chimeric genes contain a DNA sequence encoding the optimized chloroplast transit peptide (in place of the N-terminal bacterial signal peptide) as described in US Patent 5,510,471 (or Reissued US Patent RE 036449) resulting in targeting to the plastids or a signal peptide causing targeting to the cell wall. Other signal peptides effective in plant cells can similarly be used, preferably the signal peptide encoded by the alpha-amylase 3 gene of rice (Sutliff et al., 1991, Plant Molec. Biol. 16, 579-591), the signal peptide encoded by the ferredoxin NADP+ oxidoreductase gene from spinach (Oelmueller et al., 1993, Mol. Gen. Genet. 237, 261-272), or the signal peptide

encoded by the proteinase inhibitor II gene of potato (Keil et al., 1986, Nucl. Acids Res. 14, 5641-5650).

Corn cells are stably transformed with the above isp1A and isp2A chimeric genes using
5 Agrobacterium-mediated transformation (Ishida et al., 1996, Nature Biotechnology 14, 745-750;
and U.S. Patent No. 5,591,616), protoplast transformation as described in US patent 5,792,936,
or by electroporation using wounded and enzyme-degraded embryogenic callus, as described
in WO 92/09696 or US Patent 5,641,664 (all of these above references are incorporated herein
by reference). Corn plants are regenerated from the transformed cells and are selected for
10 optimal expression levels in roots and for adequate overall agronomic characteristics.

The thus obtained transgenic corn plants producing the above ISP proteins of the invention
cause significant mortality to Diabrotica virgifera larvae attempting to feed on such plants.

In accordance with this invention, stably transformed corn plants are also obtained containing
15 several DNA constructs, so that the corn plant can express one or both the ISP proteins of the
invention in its cells under control of the suitable regulatory control regions, together with an
appropriate marker gene, preferably a herbicide resistance gene. In these DNA constructs, a
preferred 3' transcription termination and polyadenylation sequence is a 215 bp fragment
containing the 3' transcription termination and polyadenylation sequences obtained from
20 Cauliflower Mosaic virus (Oka et al., 1981, J. Mol. Biol. 147, 217-226). In some constructs, a 5'
untranslated leader sequence can be added, preferably those chosen from the leader
sequences of a cab22 gene from Petunia (Harpster et al., 1988, Mol. Gen Genetics 212, 182-
190) or the zrp2 gene (Held et al, 1997, Plant Molecular Biology 35, 367-375, Genbank
accession number U38790). Preferred promoters in the constructs are promoter-effective parts
25 of the 35S promoter (e.g., Odell et al., 1985, Nature 313, 810-812) or zrp2 promoters (Held et
al, 1997, Plant Molecular Biology 35, 367-375, Genbank accession number U38790). The

constructs can also contain a signal peptide effective in plant cells besides the above optimized chloroplast transit peptide, namely the signal peptide of the alpha-amylase 3 gene of rice (Sutliff et al., 1991, Plant Molec. Biol. 16, 579-591), the signal peptide of the ferredoxin NADP+ oxidoreductase from spinach (Oelmueller et al., 1993, Mol. Gen. Genet. 237, 261-272), or the
 5 signal peptide from the proteinase inhibitor II of potato (Keil et al., 1986, Nucl. Acids Res. 14, 5641-5650). Transformations of plants with the ISP chimeric genes using a selection from the above preferred elements using techniques available to the person of ordinary skill in the art can be used to achieve the desired goal of the invention.

Preferably the plants also contain a gene encoding a protein conferring resistance to
 10 glufosinate or glyphosate, for which a different promoter and/or leader sequence is used, e.g. the *gos2* promoter and/or the *gos2* leader (de Pater et al., 1992, Plant J. 2, 837-844).

Needless to say, this invention is not limited to the above corn plants, transformation methods nor to the particular ISP proteins or DNA sequences used. Rather, the invention also includes
 15 any variant or equivalent of the ISP protein retaining insecticidal activity, such as a protein having substantially the amino acid sequence of the ISP proteins of the invention and having substantially the insecticidal activity of the ISP proteins. Also, any plant which is susceptible to damage by coleopteran insects, particularly corn rootworms, weevils or potato beetles, especially *Diabrotica virgifera* or *Leptinotarsa decemlineata*, preferably an insect selected from
 20 the group consisting of: *Agelastica alni*, *Hypera postica*, *Hypera brunneipennis*, *Haltica tombacina*, *Anthonomus grandis*, *Tenebrio molitor*, *Triboleum castaneum*, *Dicladispa armigera*, *Trichispa serica*, *Oulema oryzae*, *Colaspis brunnea*, *Lissorhoptrus oryzophilus*, *Phyllotreta cruciferae*, *Phyllotreta striolata*, *Psylliodes punctulata*, *Entomoscelis americana*, *Meligethes aeneus*, *Ceutorynchus* sp., *Psylliodes chrysocephala*, *Phyllotreta undulata*, *Leptinotarsa*
 25 *decemlineata*, *Diabrotica undecimpunctata undecimpunctata*, *Diabrotica undecimpunctata*

howardi, Diabrotica barberi and Diabrotica virgifera, is included in the invention as a preferred target for transformation with a DNA encoding an ISP protein.

Claims:

1. A protein comprising the amino acid sequence of the smallest active toxin of the protein
5 of SEQ ID No. 2, wherein said smallest active toxin is:
 - a. a fragment of the protein of SEQ ID No. 2, and
 - b. insecticidal to Diabrotica virgifera larvae when ingested by said larvae in combination with the protein of SEQ ID No. 4 from amino acid position 51 to 457.
- 10 2. A protein comprising the amino acid sequence of the smallest active toxin of the protein of SEQ ID No. 4, wherein said smallest active toxin is:
 - a. a fragment of the protein of SEQ ID No. 4, and
 - b. insecticidal to Diabrotica virgifera larvae when ingested by said insect in combination with the protein of SEQ ID No. 2 from amino acid position 38 to 871.
- 15 3. A protein comprising the amino acid sequence of SEQ ID No. 2 from amino acid position 38 to amino acid position 768 or 871.
4. A protein according to claim 3 comprising the amino acid sequence of SEQ ID No. 2 or
20 SEQ ID No. 10.
5. A protein comprising the amino acid sequence of SEQ ID No. 4 from amino acid position 51 to amino acid position 449 or 457.
- 25 6. A protein according to claim 5 comprising the amino acid sequence of SEQ ID No. 4 or SEQ ID No. 8.

7. A protein comprising the amino acid sequence of the protease-digestion fragment of the protein encoded by the isp1A DNA deposited at the BCCM-LMBP under accession number LMBP 4009, wherein said protease-digestion fragment is insecticidal to Diabrotica virgifera upon combined application with the protein of SEQ ID. No. 4 from amino acid position 51 to amino acid position 457.
8. A protein comprising the amino acid sequence of the protease-digestion fragment of the protein encoded by the isp2A DNA deposited at the BCCM-LMBP under accession number LMBP 4009, wherein said protease-digestion fragment is insecticidal to Diabrotica virgifera upon combined application with the protein of SEQ ID No. 2 from amino acid position 38 to amino acid position 871.
9. A protein according to claim 7, wherein said protease-digestion fragment is obtainable by treatment with coleopteran gut juice.
10. A protein according to claim 8, wherein said protease-digestion fragment is obtainable by treatment with coleopteran gut juice.
11. A DNA sequence encoding the protein of any one of claims 1, 3, 4, 7, or 9.
12. A DNA sequence encoding the protein of any one of claims 2, 5, 6, 8 or 10.

13. The DNA of claim 11, comprising an artificial DNA sequence having a different codon usage compared to the naturally occurring DNA sequence but encoding the same protein sequence.
- 5 14. The DNA of claim 12, comprising an artificial DNA sequence having a different codon usage compared to the naturally occurring DNA sequence but encoding the same protein sequence.
- 10 15. A chimeric gene comprising the DNA of claim 13 and a plant-expressible promoter region.
16. A chimeric gene comprising the DNA of claim 14 and a plant-expressible promoter region.
- 15 17. The chimeric gene of claim 15, wherein said promoter region is preferentially active in root tissue.
18. The chimeric gene of claim 16, wherein said promoter region is preferentially active in root tissue.
- 20 19. The chimeric gene of claim 15, wherein said promoter comprises the DNA sequence of SEQ ID No. 5 or 6 or a DNA hybridizing thereto under stringent hybridization conditions.
- 25 20. The chimeric gene of claim 16, wherein said promoter comprises the DNA sequence of SEQ ID No. 5 or 6 or a DNA hybridizing thereto under stringent hybridization conditions.

21. The chimeric gene of claim 15 wherein said chimeric gene further comprises a signal peptide for secretion from the cell or for targeting to a cellular organelle.
22. The chimeric gene of claim 16 wherein said chimeric gene further comprises a signal peptide for secretion from the cell or for targeting to a cellular organelle.
23. The chimeric gene of claim 21 wherein said signal peptide is a chloroplast transit peptide.
24. The chimeric gene of claim 22 wherein said signal peptide is a chloroplast transit peptide.
25. A plant cell comprising a chimeric gene selected from the chimeric gene of any of claims 15, 17, 19, 21, or 23.
26. A plant cell comprising a chimeric gene selected from the chimeric gene of any one of claims 16, 18, 20, 22, or 24.
27. A plant cell transformed to contain a chimeric gene selected from the chimeric gene of any one of claims 15, 17, 19, 21, or 23; and a chimeric gene selected from the chimeric gene of any one of claims 16, 18, 20, 22, or 24.
28. A plant cell transformed to produce the protein of any one of claims 1, 3, 4 or 7, and the protein of any one of claims 2, 5, 6 or 8.

29. A plant or a seed, comprising a chimeric gene integrated in its cells, wherein said chimeric gene is selected from the chimeric gene of any one of claims 15, 17, 19, 21, or 23.
- 5 30. A plant or a seed comprising a chimeric gene selected from the chimeric gene of any one of claims 16, 18, 20, 22, or 24.
31. The plant or seed of claim 29 which is a corn plant or seed.
- 10 32. The plant or seed of claim 30 which is a corn plant or seed.
33. A microorganism, transformed to contain DNA of claim 11 or the DNA of claim 12.
34. A process for controlling insects, comprising expressing a protein in cells of a plant, wherein said protein is selected from the protein of any one of claims 1 to 8.
- 15 35. A process for rendering a plant resistant to coleopteran insects, comprising transforming plants cells with a first chimeric gene and a second chimeric gene, and regenerating transformed plants from said cells which are resistant to insects, wherein said first chimeric gene is selected from the chimeric gene of any one of claims 15, 17, 19, 21, or 23; and said second chimeric gene is selected from the chimeric gene of any one of claims 16, 18, 20, 22, or 24.
- 20 36. A process for controlling coleopteran insect pests, comprising planting, sowing or growing in a field plants transformed with a first chimeric gene and a second chimeric gene, wherein said first chimeric gene is selected from the chimeric gene of any one of
- 25

claims 15, 17, 19, 21, or 23; and said second chimeric gene is selected from the chimeric gene of any one of claims 16, 18, 20, 22, or 24.

37. The process of claim 36, wherein said plants are corn plants.

5

38. The process of any one of claims 35, 36 or 37, wherein said insect is selected from the group consisting of: rootworms, weevils, potato beetles, *Diabrotica* species, *Anthonomus* spp., *Leptinotarsa* spp., *Agelastica alni*, *Hypera postica*, *Hypera brunneipennis*, *Haltica tombacina*, *Anthonomus grandis*, *Tenebrio molitor*, *Triboleum castaneum*, *Diadisa armigera*, *Trichispa serica*, *Oulema oryzae*, *Colaspis brunnea*, *Lissorhynchus oryophilus*, *Phyllotreta cruciferae*, *Phyllotreta striolata*, *Psylliodes punctulata*, *Entomoscelis americana*, *Meligethes aeneus*, *Ceutorynchus* sp., *Psylliodes chrysocephala*, *Phyllotreta undulata*, *Leptinotarsa decemlineata*, *Diabrotica undecimpunctata undecimpunctata*, *Diabrotica undecimpunctata howardi*, *Diabrotica barberi*, and *Diabrotica virgifera*.

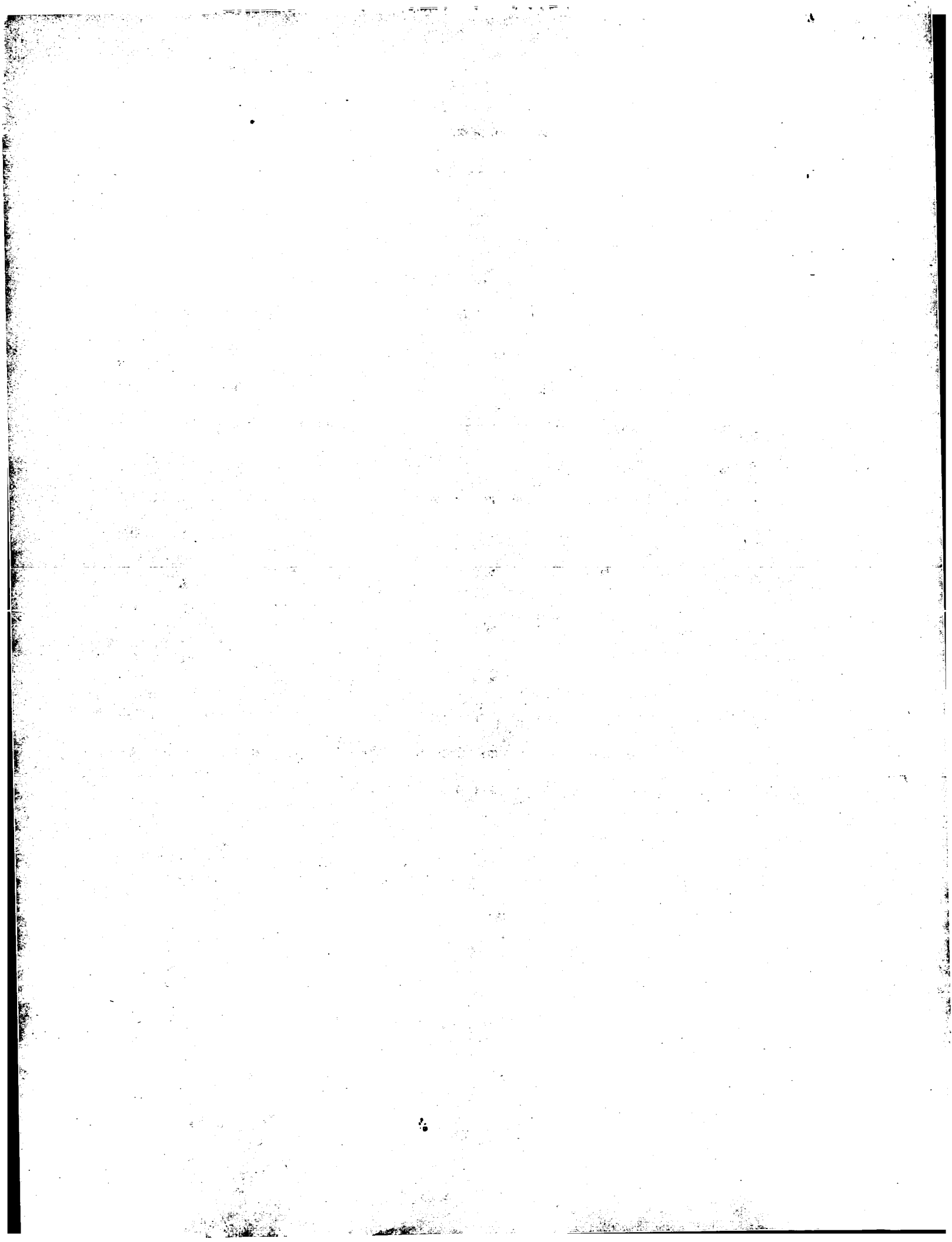
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39. A product, comprising the protein of any one of claims 1, 3, 4, 7, or 9 and the protein of any one of claims 2, 5, 6, 8, and 10 as a combined preparation for simultaneous, separate or sequential use to protect corn plants against corn rootworms.

20

40. The product of claim 39 which is a corn plant or an insecticidal composition to protect corn plants against corn rootworms.



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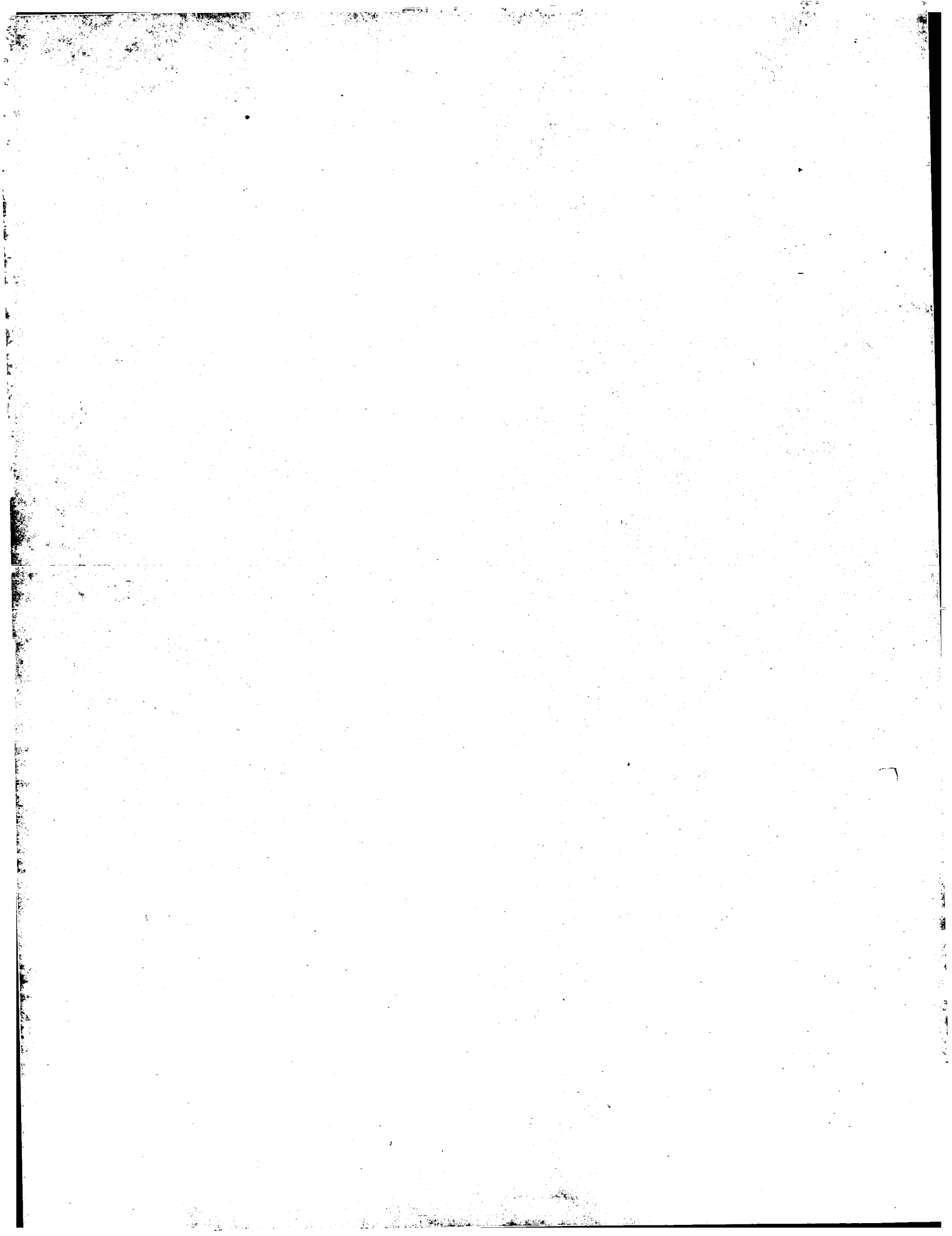
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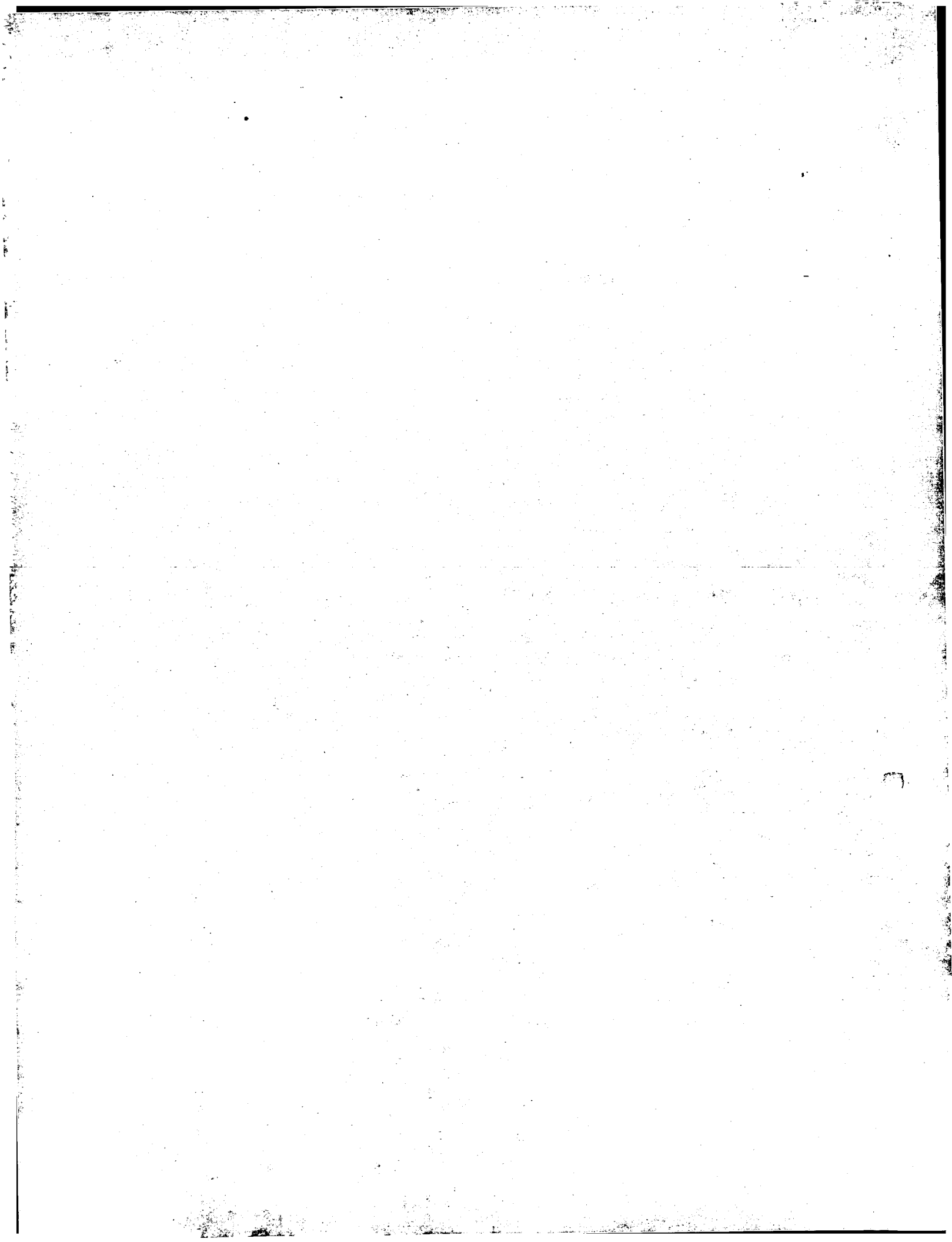
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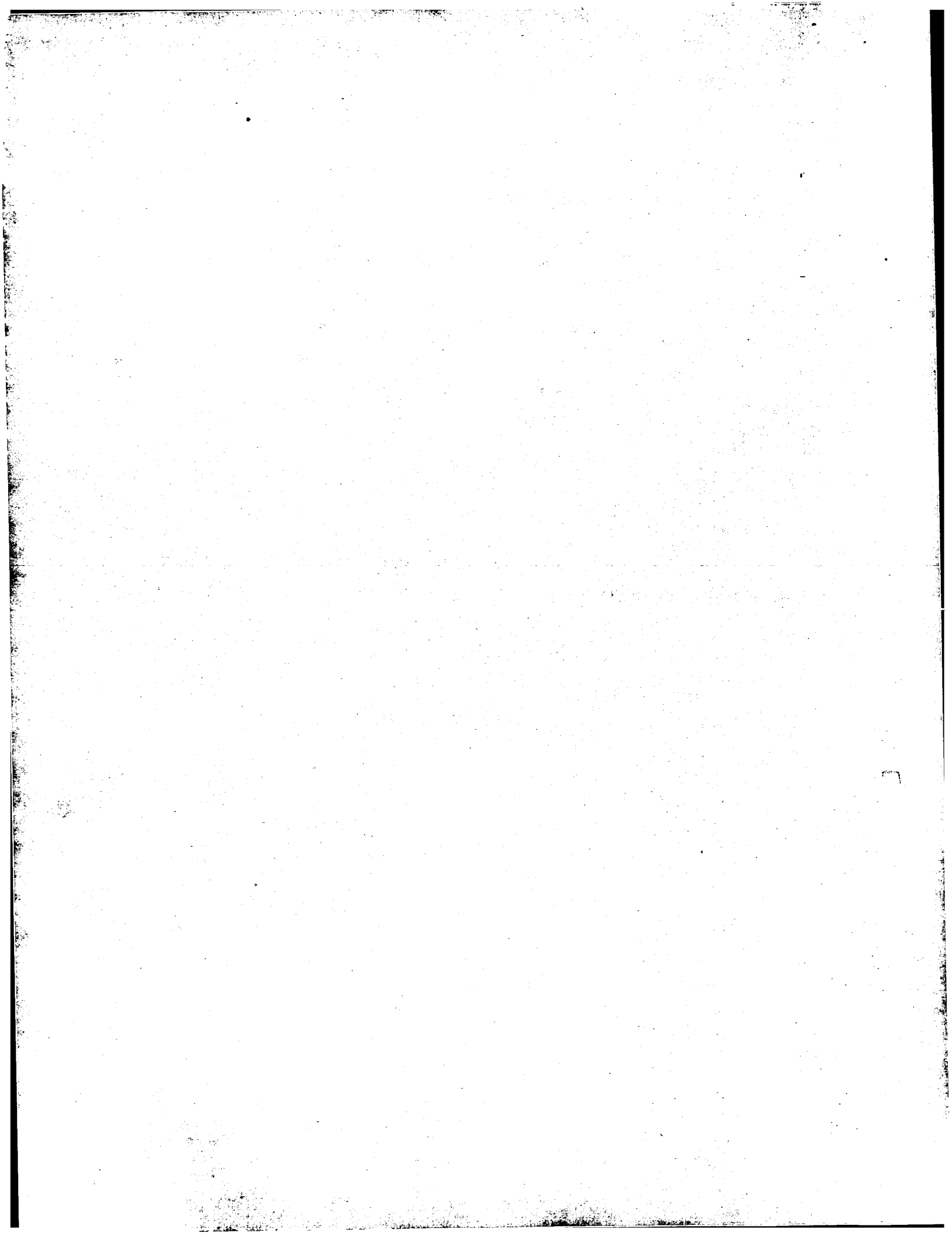
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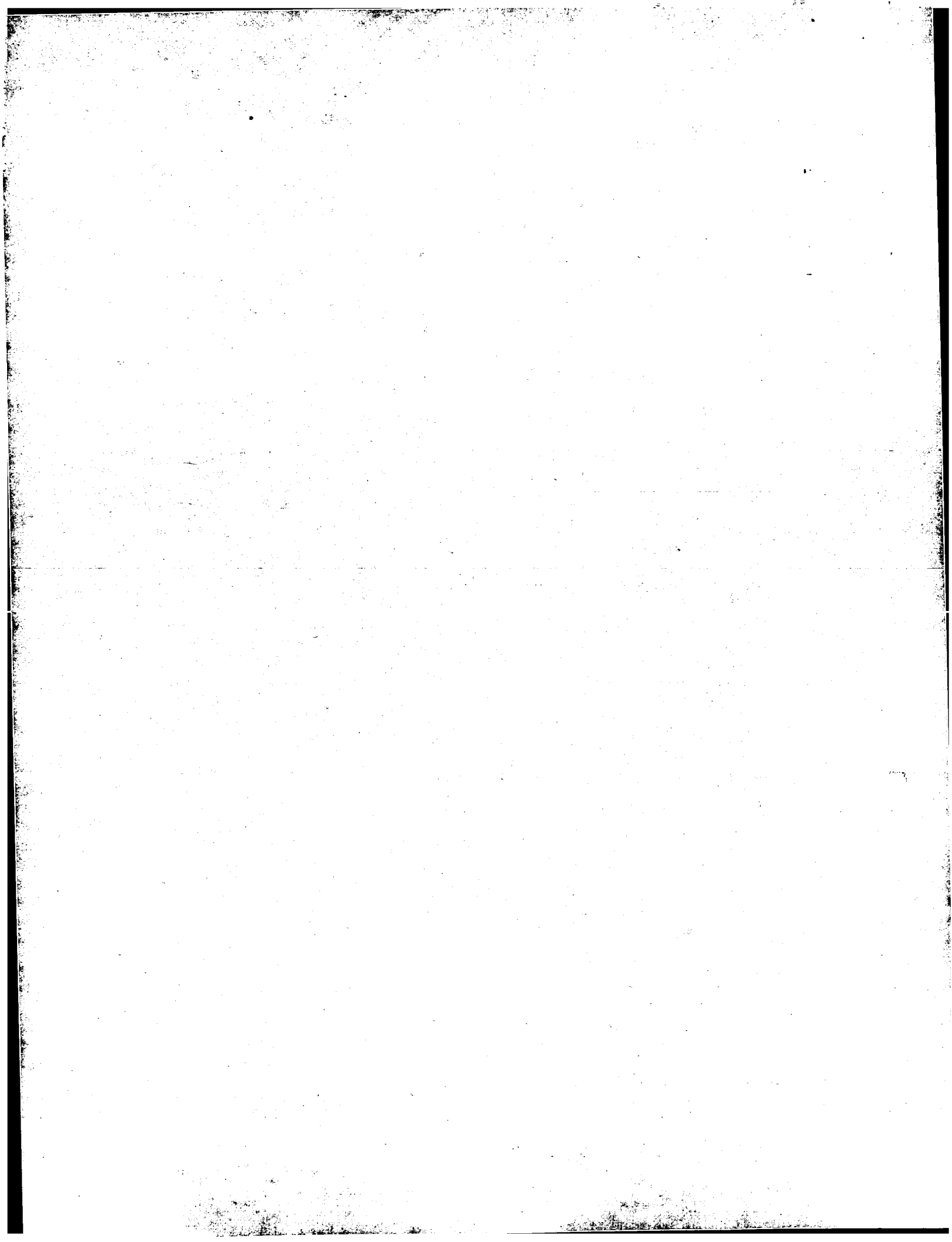
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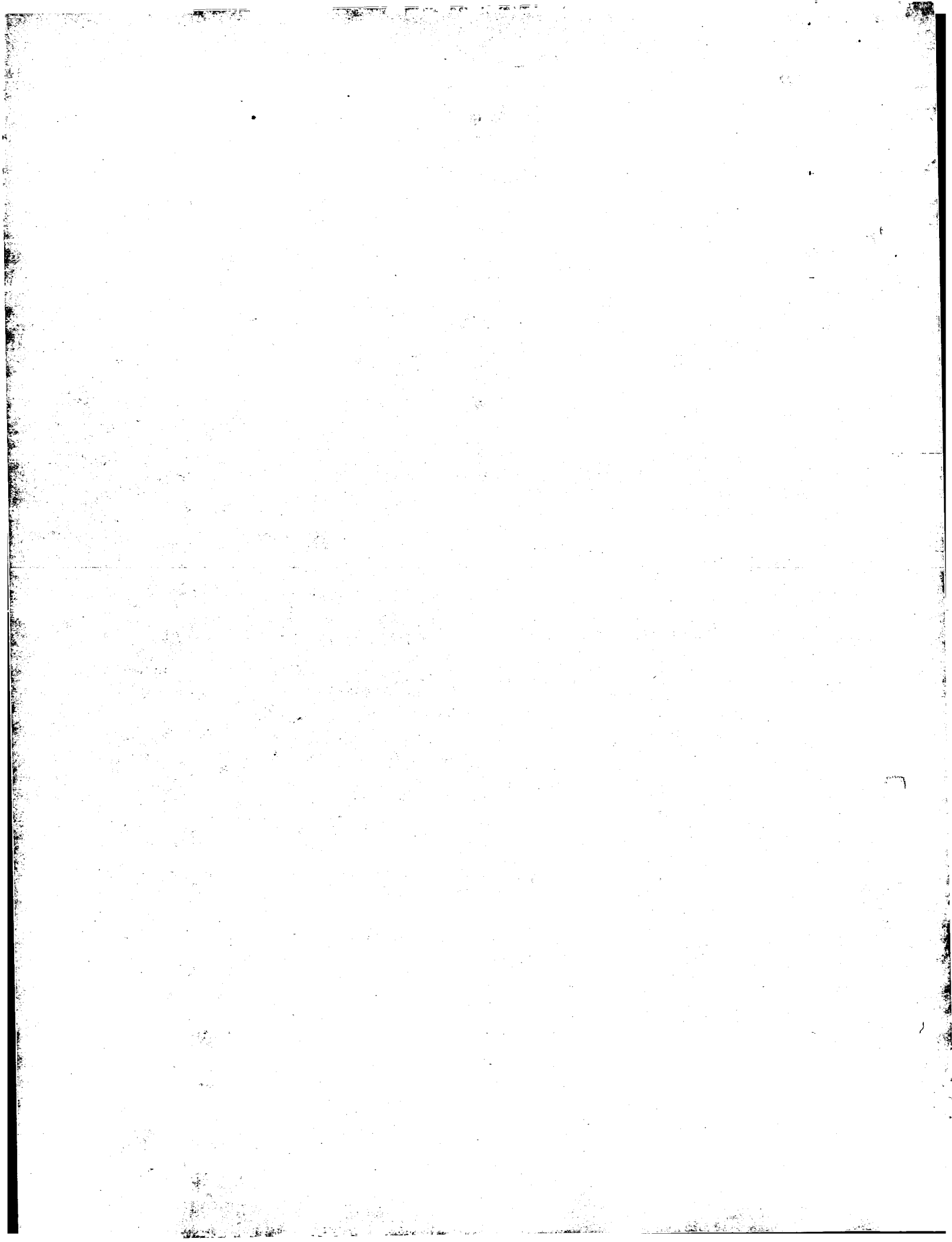
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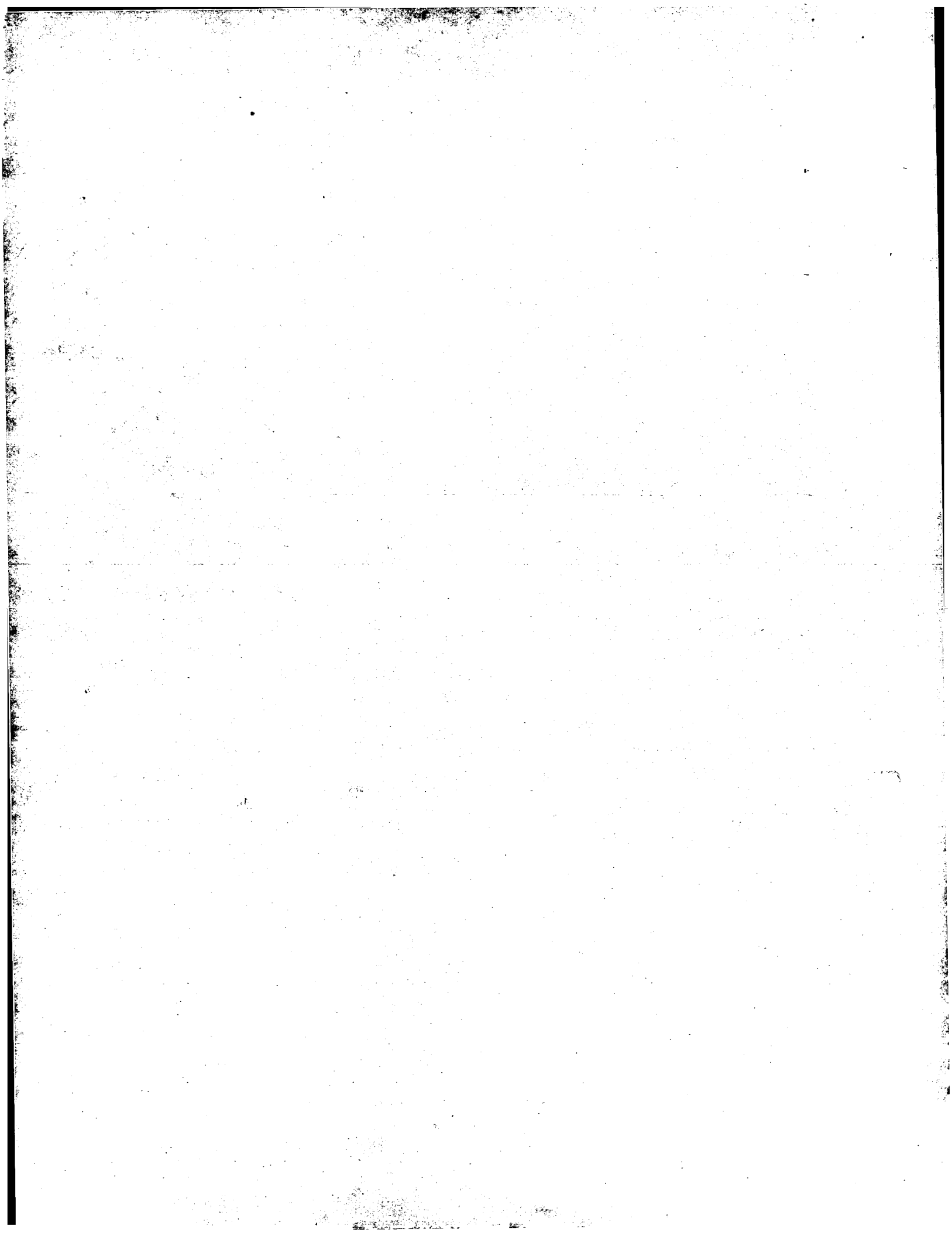
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att aag ttt gat agt tat ctt gac act cac tta act gca caa aat gtt Ile Lys Phe Asp Ser Tyr Leu Asp Thr His Leu Thr Ala Gln Asn Val 180 185 190	576
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tcg aaa gta gta aaa aaa ggt tat gaa tgt tta caa att caa gga acg Ser Lys Val Val Lys Lys Gly Tyr Glu Cys Leu Gln Ile Gln Gly Thr 245 250 255	768
cta aaa aag agt ctc gat ttt aaa aat gat att aat gct gag gct cat Leu Lys Lys Ser Leu Asp Phe Lys Asn Asp Ile Asn Ala Glu Ala His 260 265 270	816
cgt tgg ggt atg aaa aat tat gaa gga tgg gct aaa aat tta aca gat Arg Trp Gly Met Lys Asn Tyr Glu Gly Trp Ala Lys Asn Leu Thr Asp 275 280 285	864
cct caa agg gaa gct tta gat ggg tat gct aga caa gat tat aaa caa Pro Gln Arg Glu Ala Leu Asp Gly Tyr Ala Arg Gln Asp Tyr Lys Gln 290 295 300	912
ata aat gat tat tta cga aat caa ggt gga agt gga aat gaa aaa cta Ile Asn Asp Tyr Leu Arg Asn Gln Gly Gly Ser Gly Asn Glu Lys Leu 305 310 315 320	960



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 Asp Thr Gln Ile Lys Asn Ile Ser Glu Ala Leu Glu Lys Gln Pro Ile 335
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 cca gaa aat att act gtg tat aga tgg tgt gga atg gcg gaa ttt ggt 1056
 Pro Glu Asn Ile Thr Val Tyr Arg Trp Cys Gly Met Ala Glu Phe Gly 350
 340 345 350
 tat caa att agt gat cct tta cct tct tta aaa gaa atg gaa gaa aaa 1104
 Tyr Gln Ile Ser Asp Pro Leu Pro Ser Leu Lys Glu Met Glu Glu Lys 365
 355 360 365
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 Phe Leu Asn Thr Met Lys Glu Asp Lys Gly Tyr Met Ser Thr Ser Leu 380
 370 375 380
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 385 390 395 400
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 405 410 415
 gga ttt gca agt gaa aaa gaa atc ctt att gat aaa gat agt aac tat 1296
 Gly Phe Ala Ser Glu Lys Glu Ile Leu Ile Asp Lys Asp Ser Asn Tyr 430
 420 425 430
 cat att gat aaa ata aca gag gta gtc att aaa ggt gtt aag cga tat 1344
 His Ile Asp Lys Ile Thr Glu Val Val Ile Lys Gly Val Lys Arg Tyr 445
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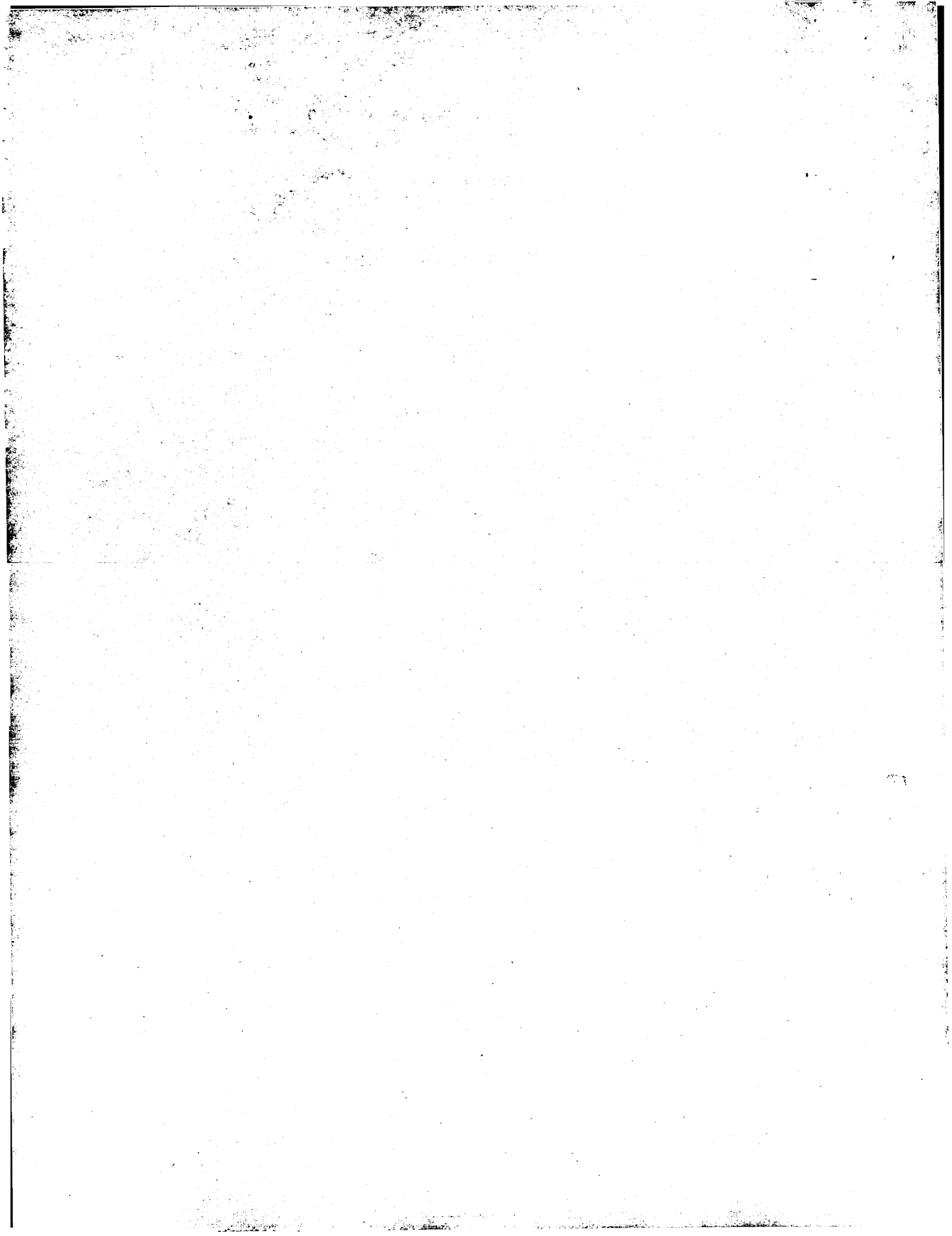
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Met Pro Ile Ser Ser Asn Val Thr Tyr Ala Tyr Gly Ser Glu Lys Val
 35 40 45



WO 01/87931

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 85 90 95

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 100 105 110

Ser Phe Glu Asp Glu Ile Lys Asp Leu Lys Glu Ile Asp Lys Met Phe
 115 120 125

Asp Lys Ala Asn Leu Ser Ser Ser Ile Val Thr Tyr Lys Asn Val Glu
 130 135 140

Pro Ser Thr Ile Gly Phe Asn Lys Pro Leu Thr Glu Gly Asn Thr Ile
 145 150 155 160

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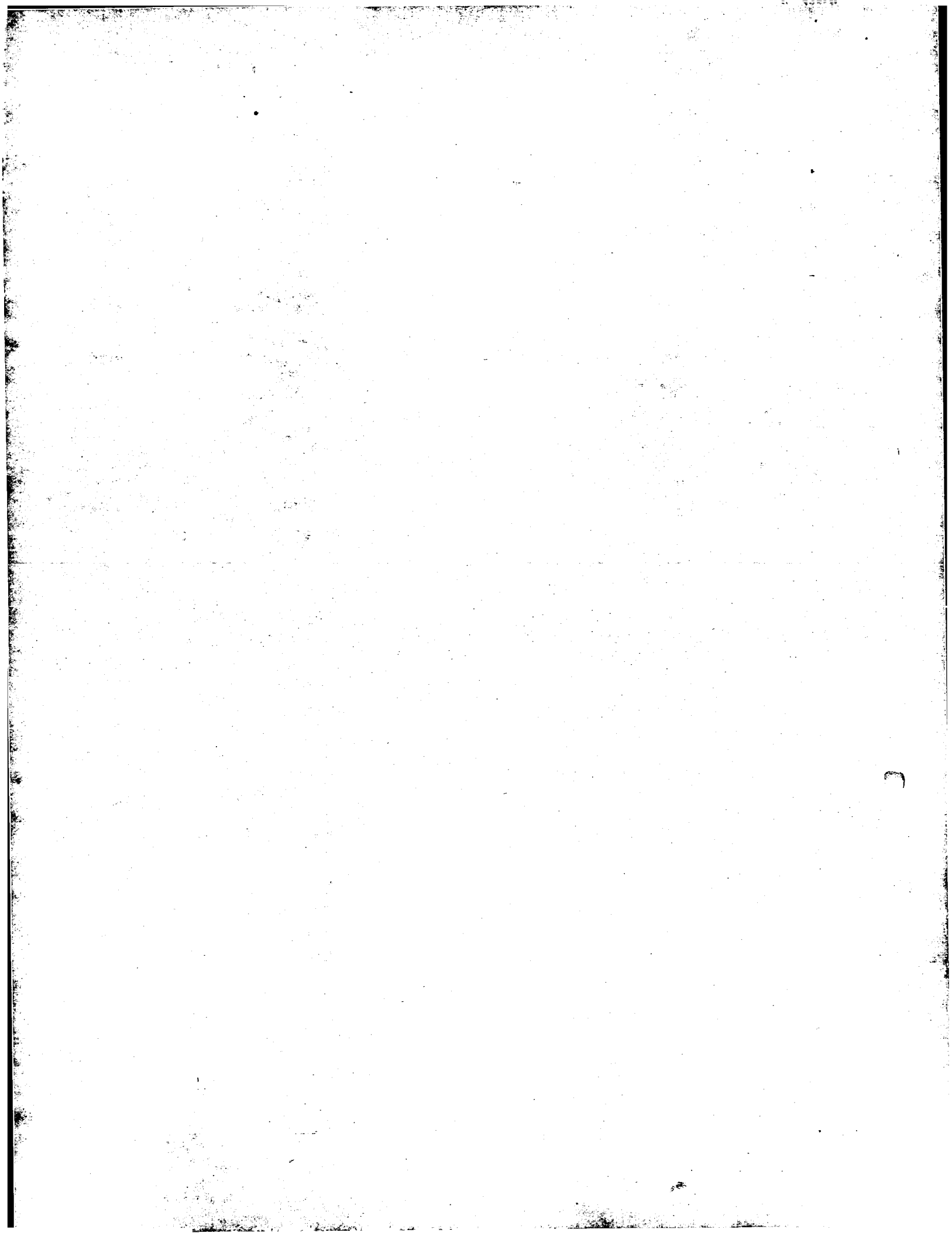
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Tyr Lys Met Leu Ile Asp Asn Gly Tyr Val Leu His Val Asp Asn Ile
 225 230 235 240

Ser Lys Val Val Lys Lys Gly Tyr Glu Cys Leu Gln Ile Gln Gly Thr
 245 250 255

Leu Lys Lys Ser Leu Asp Phe Lys Asn Asp Ile Asn Ala Glu Ala His
 260 265 270



Arg Trp Gly Met Lys Asn Tyr Glu Gly Trp Ala Lys Asn Leu Thr Asp
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Pro Gln Arg Glu Ala Leu Asp Gly Tyr Ala Arg Gln Asp Tyr Lys Gln
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Ile Asn Asp Tyr Leu Arg Asn Gln Gly Gly Ser Gly Asn Glu Lys Leu
 305 310 315 320

Asp Thr Gln Ile Lys Asn Ile Ser Glu Ala Leu Glu Lys Gln Pro Ile
 325 330 335

Pro Glu Asn Ile Thr Val Tyr Arg Trp Cys Gly Met Ala Glu Phe Gly
 340 345 350

Tyr Gln Ile Ser Asp Pro Leu Pro Ser Leu Lys Glu Met Glu Glu Lys
 355 360 365

Phe Leu Asn Thr Met Lys Glu Asp Lys Gly Tyr Met Ser Thr Ser Leu
 370 375 380

Ser Ser Glu Arg Leu Ser Ala Phe Gly Ser Arg Lys Phe Ile Leu Arg
 385 390 395 400

Leu Gln Val Pro Lys Gly Ser Thr Gly Ala Tyr Leu Ser Ala Ile Gly
 405 410 415

Gly Phe Ala Ser Glu Lys Glu Ile Leu Ile Asp Lys Asp Ser Asn Tyr
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<211> 2658

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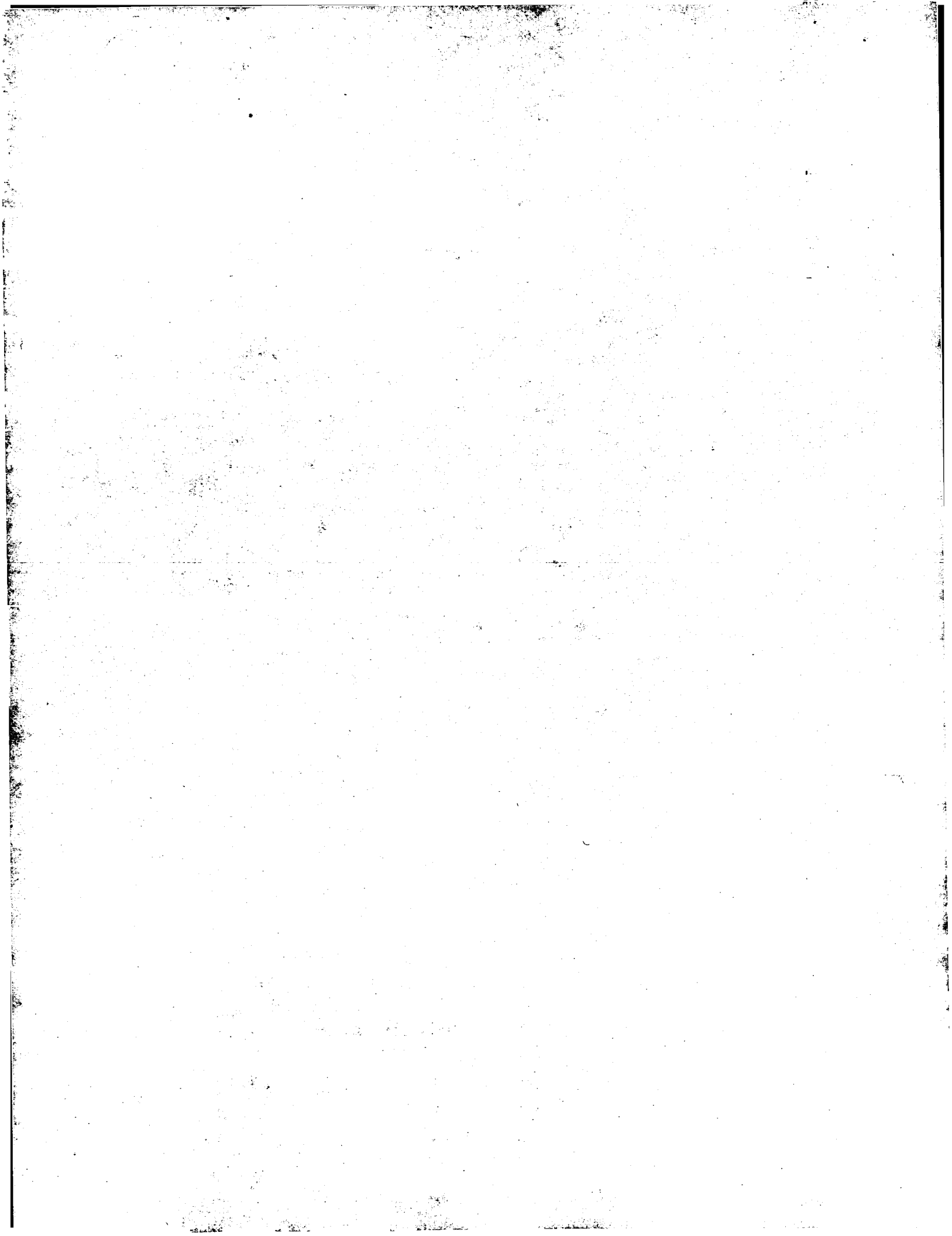
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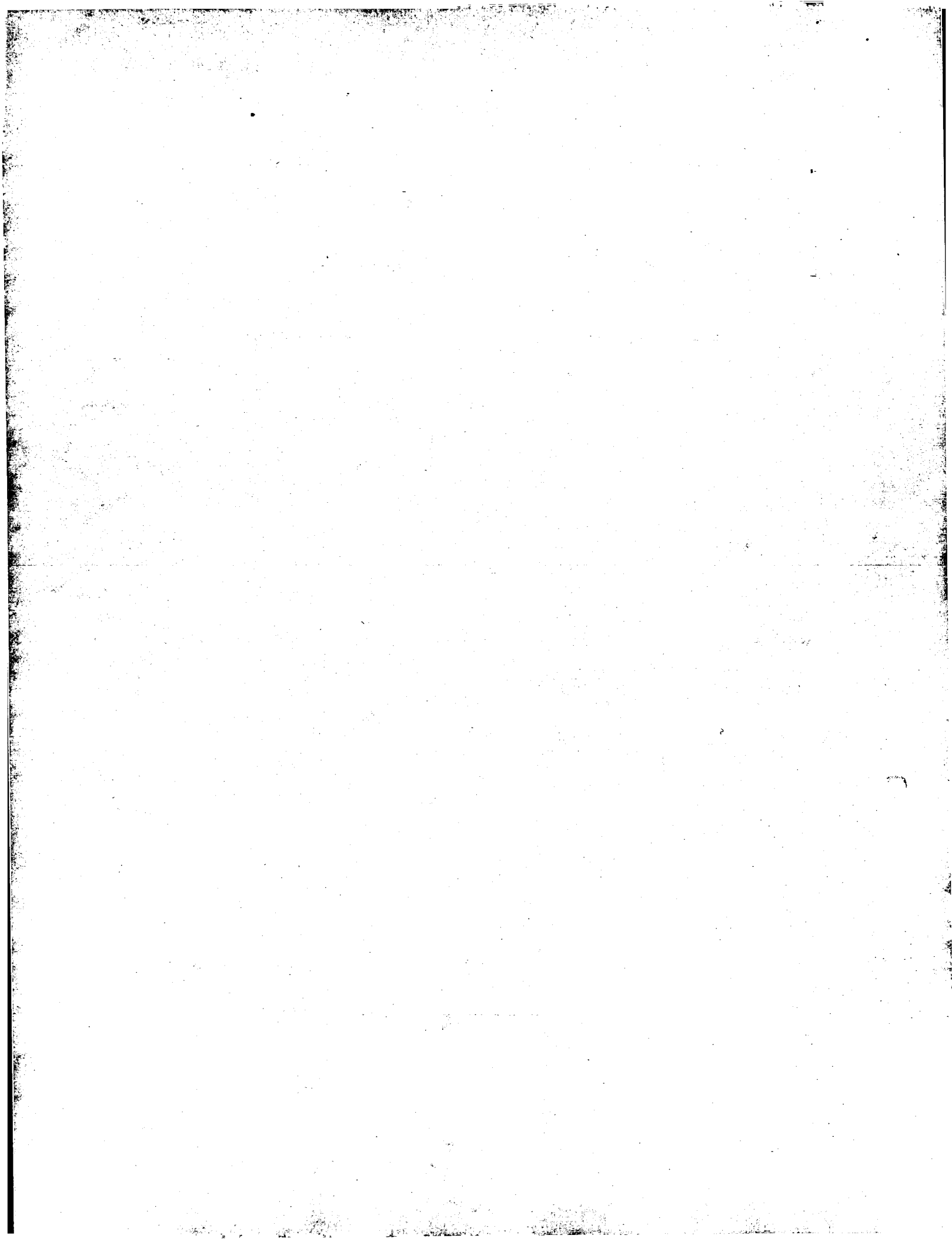
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<223> Nucleotide at position 21 is "n" wherein "n" = any nucleotide



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 tttgtgttg atacttatgt ctgtgatgat atatgtgat tatatatgt atgtatatgt 480
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 gaagtcgttg ccaatgtatt gttcgtgag gtctctttgt caagtattac actcggcaaa 960
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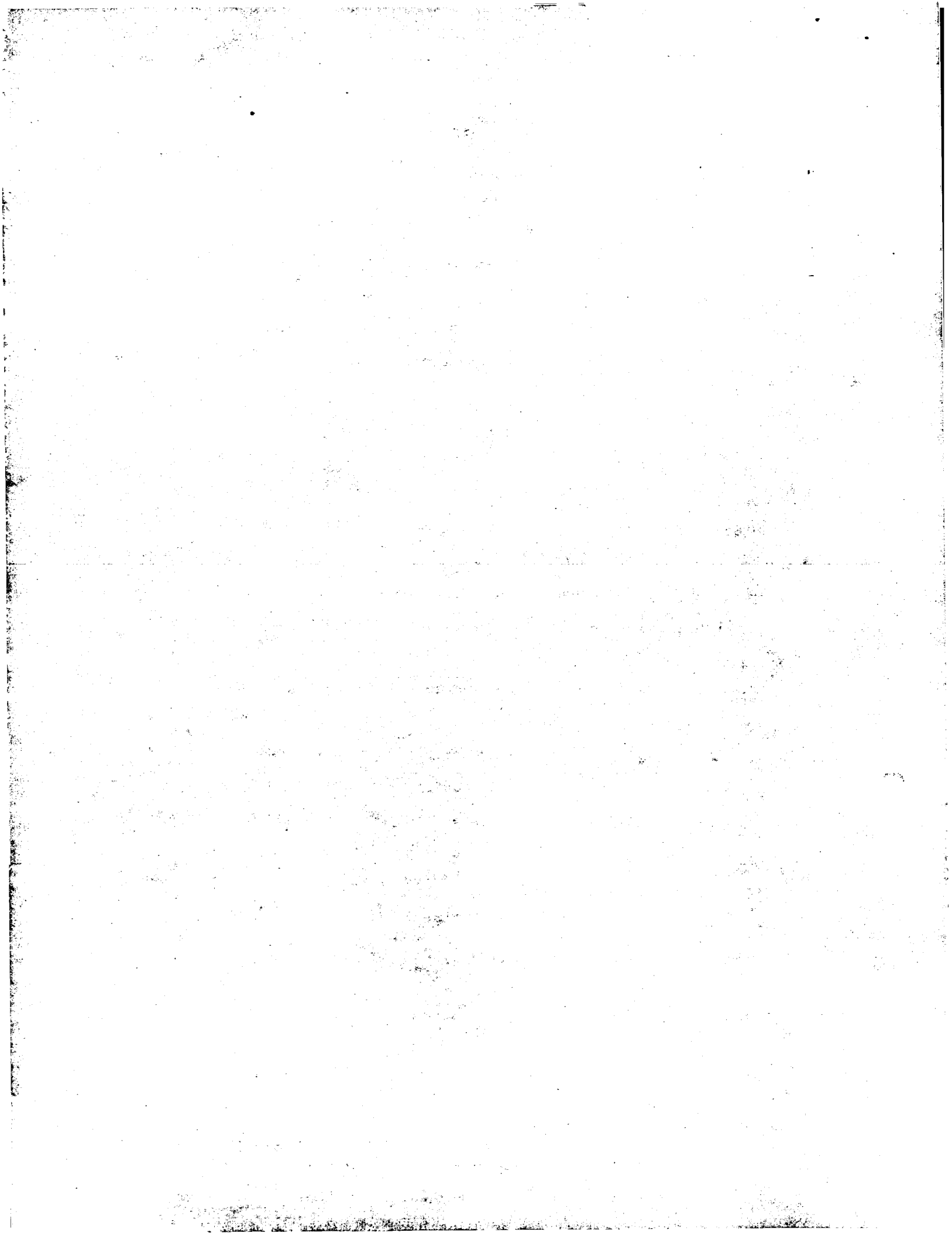
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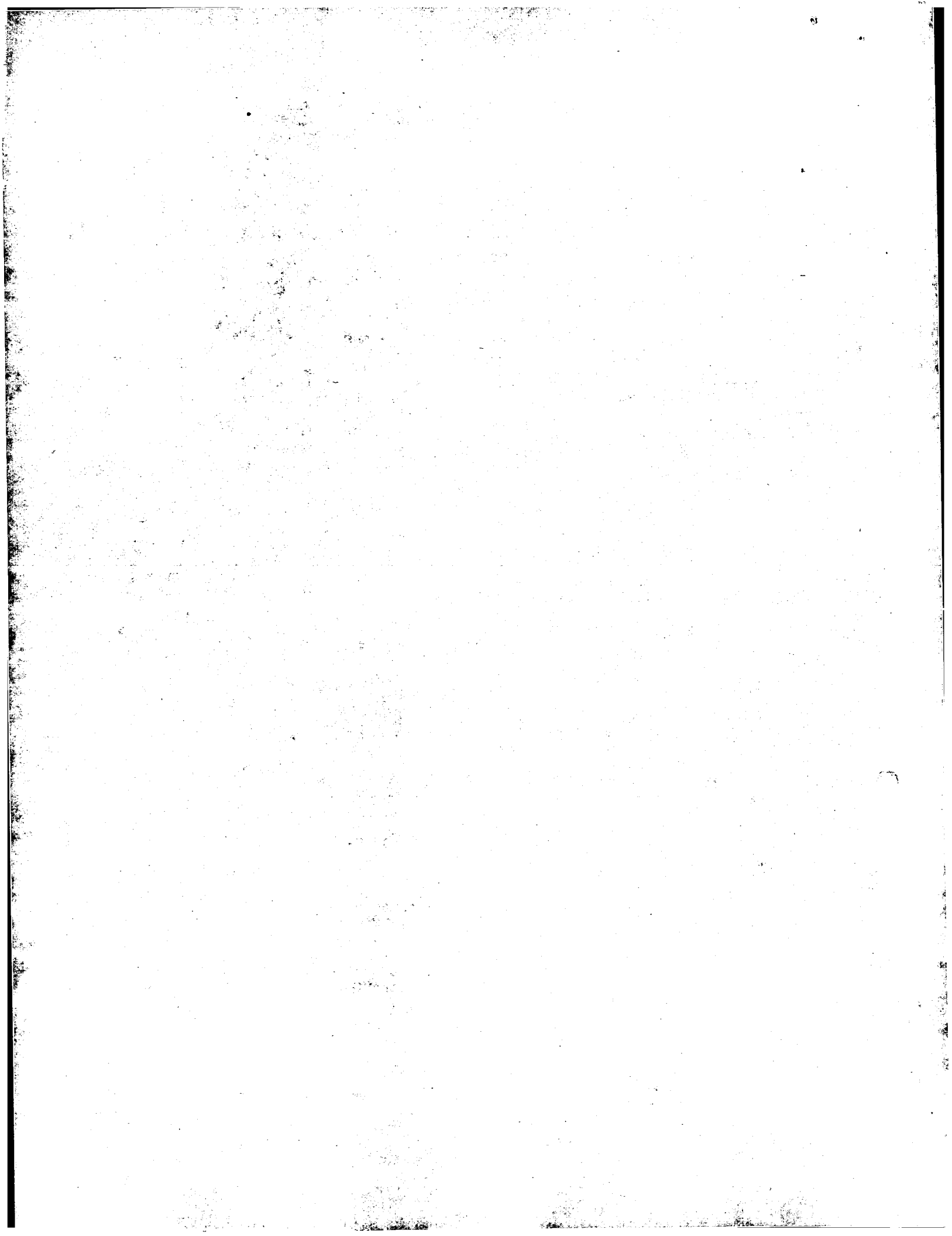
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Lys Leu Thr Val Thr Glu Lys Thr Arg Met Asn Asn Phe Leu Asp Asn
35 40 45
aag aac gac atc aag aag aac tac aag gag atc acc ttc agc atg gct 191
Lys Asn Asp Ile Lys Lys Asn Tyr Lys Glu Ile Thr Phe Ser Met Ala
50 55 60

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WO 01/87931

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WO 01/87931

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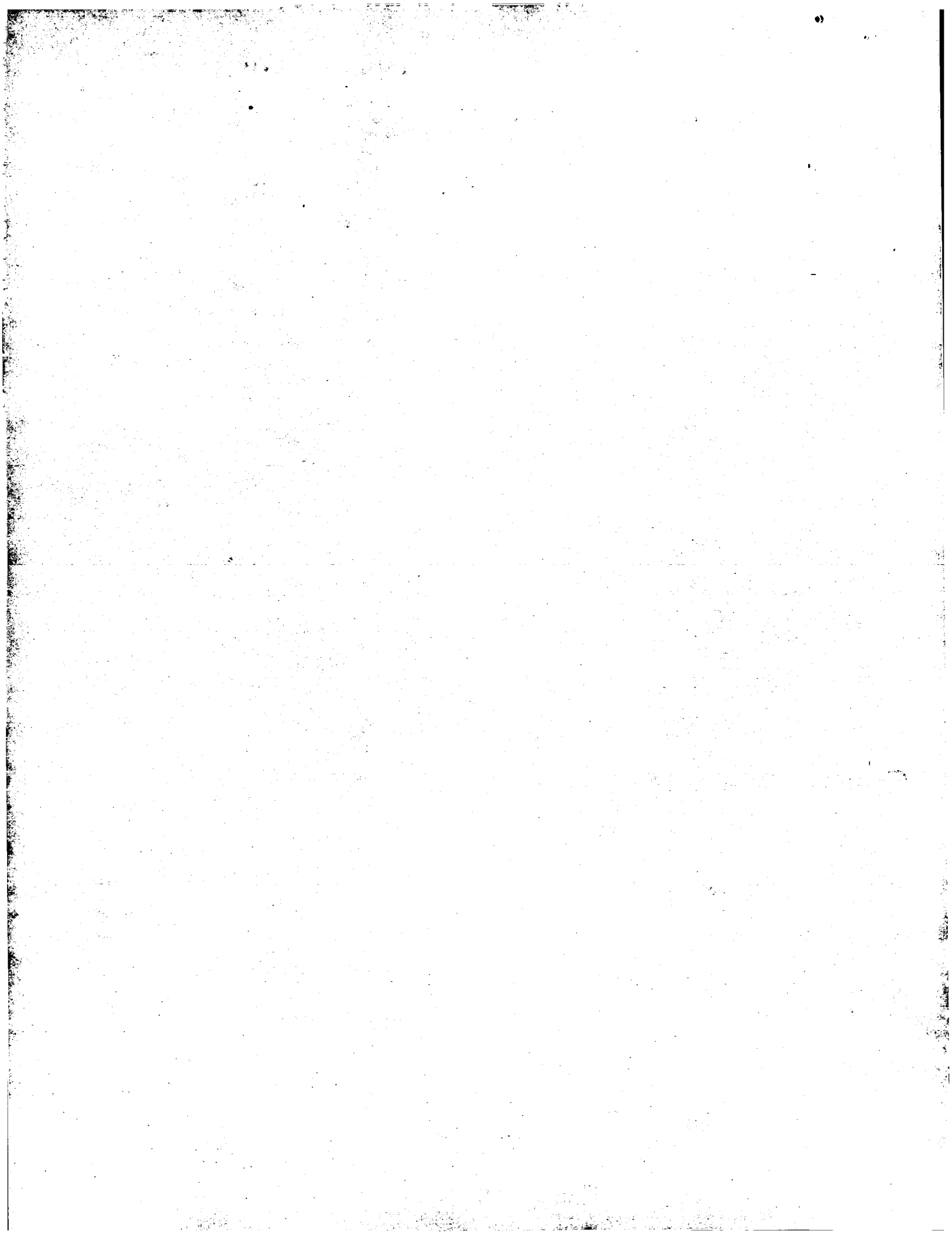
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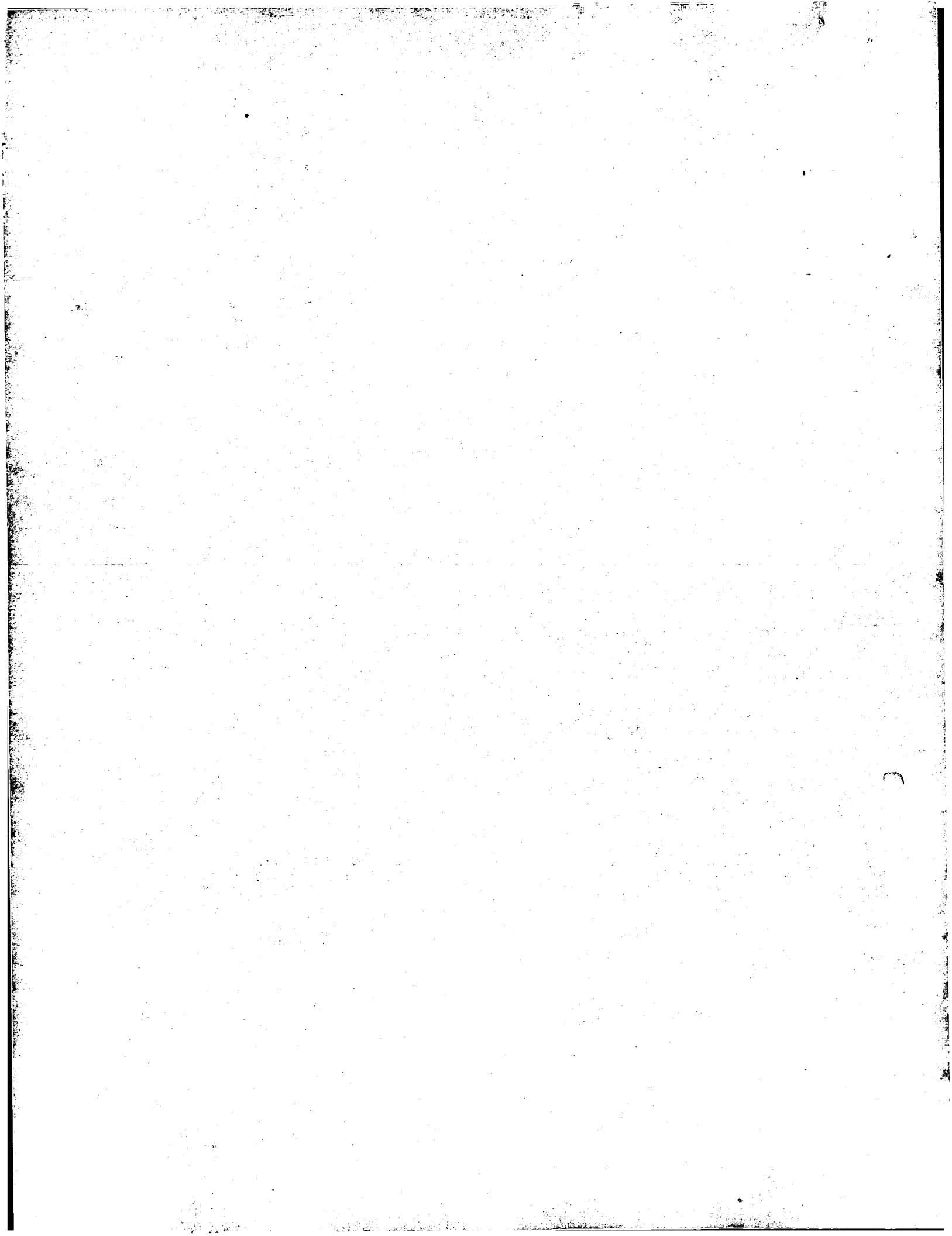
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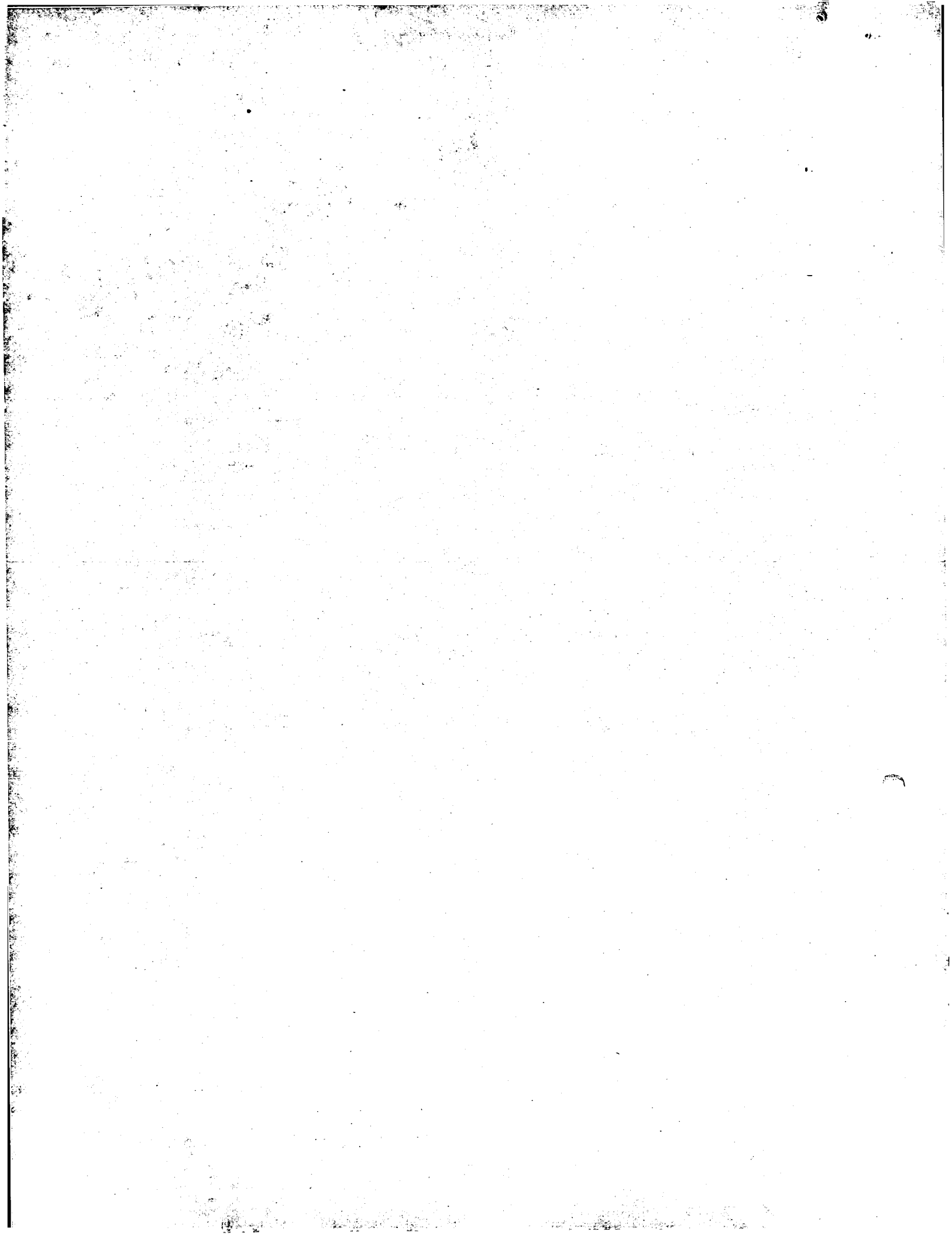
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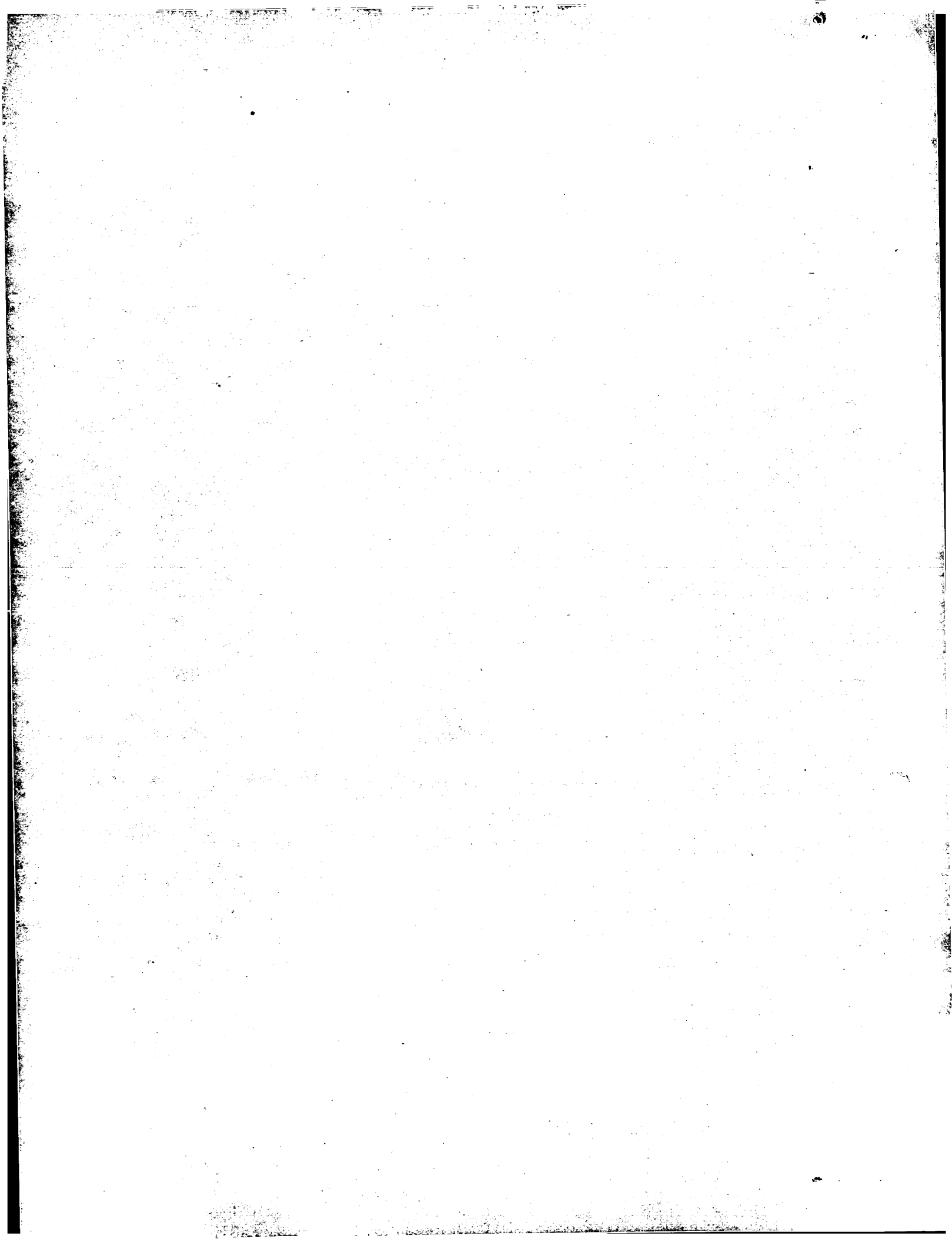
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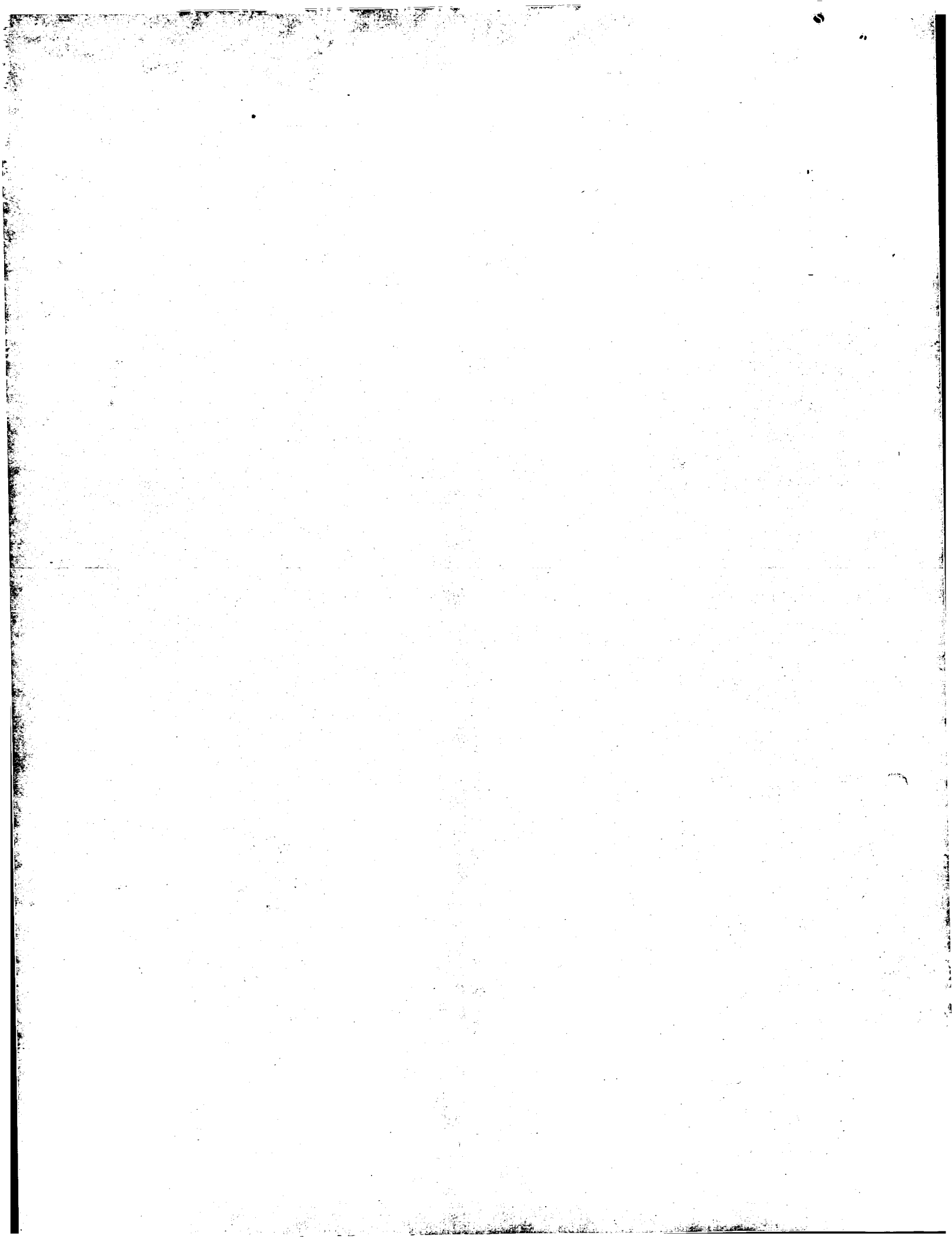
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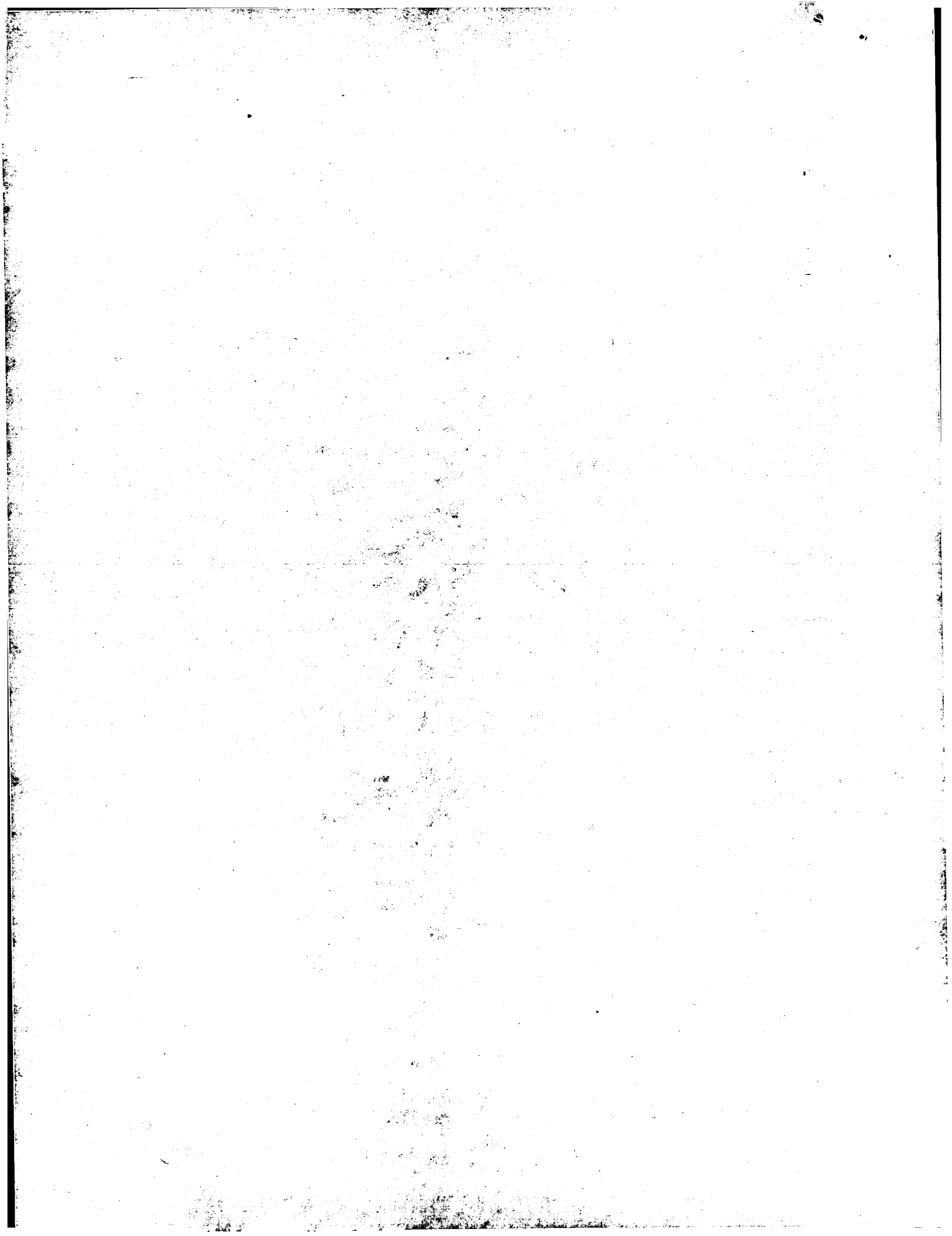
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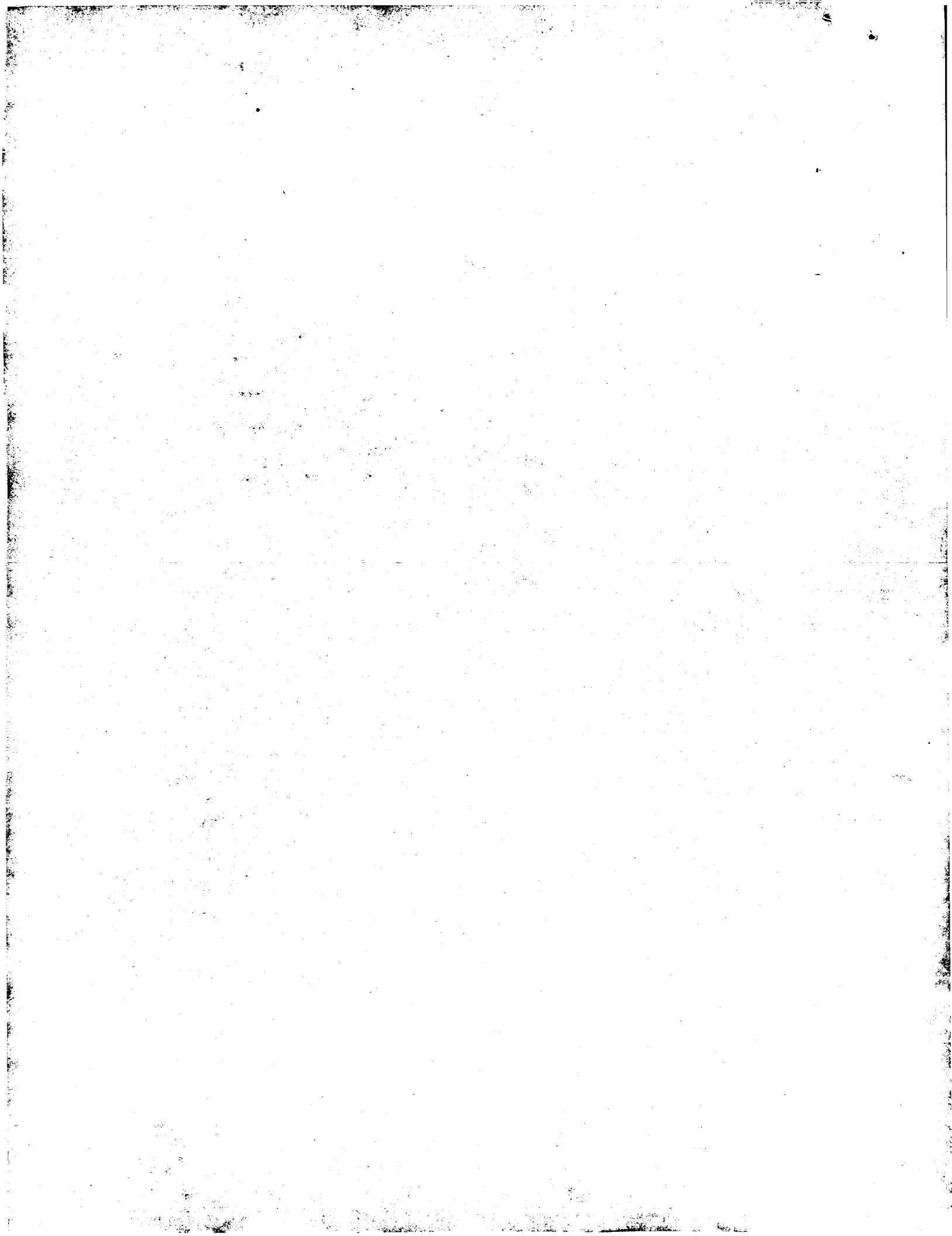
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